COMPARATIVE ANALYSIS OF FACTORS INFLUENCING MALE REPRODUCTIVE SUCCESS IN SYMPATRIC FRESHWATER TURTLES

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ABSTRACT

COMPARATIVE ANALYSIS OF FACTORS INFLUENCING MALE REPRODUCTIVE SUCCESS IN SYMPATRIC FRESHWATER TURTLES

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Male reproductive success (RS) is primarily constrained by mate number. However, when females vary in reproductive quality (i.e., reproductive frequency, clutch size, egg size), female quality can contribute substantially to male reproductive success. I used a comparative inter-specific approach, to document sources of variation in male reproductive success in three species of freshwater turtles (Painted turtles (*Chrysemys picta*), Blanding's turtles (*Emydoidea blandingii*) and Snapping turtles (*Chelydra serpentina*). Data were collected and analyzed using extensive background information from a long-term study on the life history and demography of all three species. Long-term studies (1953-2007) on the E.S. George Reserve in Michigan allowed analyses to be preformed based on females of known age, size, and reproductive history. In all three species, larger and older females were of higher reproductive quality than were smaller or younger females, suggesting that males would benefit by mating with these females compared to mating indiscriminately. Using multi-locus microsatellite genotypes and paternity analysis, I examined offspring (Painted turtles N = 1065 from 171 clutches; Blanding's turtles N = 772 from 98 clutches, Snapping turtles N = 1064 from 63 clutches) collected over four years

(Painted and Snapping turtles) and 8 years (Blanding's turtles) from nests of known females. Incidence of multiple paternity was variable among years in all three species (Painted turtles, 6.1% - 30.0%; Blanding’s turtles, 15.4% - 55.6%; Snapping turtles, 44.4% - 61.5%) and was positively associated with female age in all three species. Sperm from the same male(s) fertilized successive clutches (repeat paternity) among years in all three species. Reproductive frequency contributed substantially to male reproductive success. Paternity analyses conducted in Painted and Blanding's turtles revealed that the number of clutches sired represented a significant component to male reproductive success. Mate number was not a substantial source of variation of male reproductive success in Painted turtles, as few males mated with more than one female. In Blanding's turtles mate number and clutch number were significantly associated with male reproductive success. Female reproductive frequency increased with increasing age,. Additionally, older females are more likely to have multiple males sire offspring within a clutch suggesting that older females were comparatively more attractive to males or more receptive to mating attempts by multiple males. Clutches of eggs from older females were more genetically diverse (lower coancestry) that clutches of younger females. Therefore, older females contribute disproportionally to population levels of genetic diversity. For species such as Blanding's turtles that make extensive use of terrestrial habitats, we found that older females were more likely to have offspring sired by males from other residence wetlands than do younger females. Results highlight the need to protect corridors that facilitate movement and gene flow among permanent wetlands. The presence of age-specific effects on female reproductive quality in all three species highlight the importance of management strategies that reduce adult mortality rates.

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INTRODUCTION

INTRODUCTION

Behavioral ecological research can be enhanced when life-history information is available (Krebs and Davies 1997; Sih and Bell 2008; Bassar et al. 2010). Similarly, life-history studies also benefit from behavioral ecology studies because information on behaviors can identify sources of variation in body size and age specific reproductive success, which is a central focus of life-history studies. Life history and behavioral ecology studies focus on females, in part because of the comparative ease of observation, and because population levels of recruitment are mediated by female reproductive success. Detailed information on male reproductive success is rarely available, particularly for poikilothermic vertebrates, because paternity cannot be inferred from direct observation alone, and mating behaviors of many poikilothermic vertebrates takes place out of view (e.g., underwater). However, the importance of including information on male reproductive success in life-history studies has recently been demonstrated (Kokko and Mappes 2005, Kokko et al. 2008).

The study of male reproductive success of three species of long-lived turtles on the University of Michigan's E. S. George Reserve (ESGR) provides two important aspects to studies of mating systems. First, results from behavioral ecology studies are enhanced by detailed age-specific reproductive information on individuals being studied. For many long-lived organisms, accurate age-specific reproductive information is unavailable due to the inherit difficulties and costs associated with conducting long-term studies. Secondly, more primitive and less complex mating systems are the foundations for the evolution of complex systems. The results from my research should provide a basis for the interpretation of comparatively more complex mating systems that are complicated by factors such as social interactions, parental care, and improved access to resources.

My dissertation research uses genetic techniques to document sources of variation in male reproductive success in syntopic populations of three species of freshwater turtles (Midland Painted turtles, *Chrysemys picta marginata*; Blanding's turtles, *Emydoidea blandingii*; and Snapping turtles, *Chelydra serpentina*); see the summary of traits of the three species below (Table 0.1). The study of male reproductive success was conducted within the context of a long-term study on life history and demography initiated by Owen Sexton (1953-1957), continued by Henry Wilbur (1968-1972, Donald Tinkle (1975-1979), and Justin Congdon (1975-2007). Long-term data that quantify sources of variation in female reproductive quality (i.e., age- and size-specific clutch size, clutch frequency, and egg size for all three species, and individual-based frequencies of within-year second clutch production for Painted turtles) allows quantification of the relative importance of mate number and female reproductive qualities to male reproductive success.

In Chapter 1, entitled "Variation in Female Reproductive Quality and Reproductive Success of Male Painted Turtles (*Chrysemys picta marginata*)" the primary objective was to determine whether females primarily use stored sperm to fertilize second clutches within a year as suggested by Gist and Congdon (1998). I documented how female quality can influence male reproductive success by quantifying stored sperm use in intra-seasonally iteroparous females. (Chapter 1 is currently in press in the Canadian Journal of Zoology (accepted August 2011).

In Chapter 2, entitled " Female quality affects male Painted turtle (*Chrysemys picta marginata*) reproductive success" I quantified the relative importance of mate number and quality to male reproductive success based on genetically determined parentage estimated from offspring of nests collected over 4 years. I tested the following predictions: 1) mate number contributes significantly to male reproductive success; 2) female quality contributes substantially to male reproductive success; and 3) high-quality (e.g., older or larger) females will have a higher incidence of multiple paternity.

In Chapter 3, entitled "Mating system, male reproductive success, and population connectivity of Blanding's turtles (*Emydoidea blandingii*)" I characterized mating behaviors influencing male reproductive success, and quantified the degree of spatial and genetic structuring of threatened Blanding’s turtles. I quantified components of variation in male reproductive success in relation to: 1) number of mates, 2) mate quality (clutch size, clutch frequency, egg size), 3) occurrence of multiple paternity 4) the number of eggs a male sired per clutch, 5) repeat paternity (via stored sperm or remating), and 6) age and body size of both sexes of adults. I also quantified the proportion of successful matings among individuals from different resident wetlands on and off of the ESGR. Mating pair data were used to quantify propensities for turtles to mate with individuals outside their residence wetlands to quantify gene flow.

In Chapter 4, entitled " Factors influencing mating systems and male reproductive success in freshwater turtles: A comparative analysis" I examined patterns of reproduction among the three species. I tested hypotheses regarding factors associated with incidence of multiple paternity and repeat paternity within the context of the species similarities and differences in life history and demography. Factors associated with male reproductive success were compared between the two species of Emydid turtles (Painted turtles and Blanding's turtles). I did not have sufficient power for paternity analysis of Snapping turtles. The three species are sympatric, and sampled over the same time periods to allow inter-annual variation to be compared among the three species with respect to variation in female reproductive quality.

The research on three species of freshwater turtles provides opportunities to compare and generalize findings among species to quantify how fluctuations in environmental and demographic environments affect traits associated with reproductive success. The three turtle species present a unique opportunity to contrast components of male reproductive success, test for evidence of assortative mating, and aspects of the species mating system in general for species that differ in adult sex ratio, population size, age- and size-specific variation in reproductive quality, age at maturity and clutch size. Comparisons have here-to-fore not been possible for long-lived poikilothermic vertebrates, and thus have the potential to have a major impact on evolutionary and behavioral ecology fields.

APPENDIX 0

Table 0.1. A general description of traits and duration of nesting seasons of three freshwater turtle species.

Species	Mean body size of females (mm)	Larger sex	Adult sex ratio	Min. age at maturity of females	Longevity (years)	Clutch size	Annual clutch frequency mean (min-max)	Egg size/ body size relationship	Mean duration of nesting season (days)
Painted turtles	136	female	2.3 M /F	7	~ 50	6	1.3 (0 – 3)	Strong	37
Blanding's turtles	196	equal	Equal	14	>75	10	0.8 (0 – 1)	Weak	26
Snapping turtles	250	male	Equal	11	> 55	23	0.85 (0 – 1)	Weak	18

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CHAPTER 1: VARIATION IN FEMALE REPRODUCTIVE QUALITY AND REPRODUCTIVE SUCCESS OF MALE PAINTED TURTLES (*CHRYSEMYS PICTA MARGINATA*)

Abstract

Although mate number is perceived to be the primary factor affecting male reproductive success in polygynous systems, differences in female reproductive qualities may also influence variation in male reproductive success. We combined 32 years of data on variation in reproductive qualities (clutch size and clutch frequency) of female painted turtles (*Chrysemys picta marginata* Agassiz, 1857) with genetic data on patterns of repeated paternity (i.e., stored sperm use) and multiple paternity to examine the potential influence on male reproductive success. Over 24 years (1983-2006), the number of reproductive females each year averaged 84 (min-max = 62-106) and on average 23% (min-max = 6%-40%) produced two clutches (intra-seasonally). Among females with reproductive histories spanning 5-24 years (*N* = 167), 26% of individuals produced only one clutch annually, whereas 74% produced two clutches within a season. Among just intra-seasonally iteroparous females, second-clutch production varied from 7%-50%. Repeated paternity was observed in 97.5% of 40 paired clutches and 44% of 9 among-year comparisons of clutches from consecutive years. The frequent use of stored sperm to fertilize sequential clutches within and potentially among years can substantially increase a male's reproductive success, particularly if males can base mating decisions on phenotypic characteristics correlated with female quality.

Introduction

Life-history studies have primarily focused on age-specific traits of females, but complete understanding of the evolution of life histories will require knowledge of how interactions between males and females influence the way each sex attempts to maximize fitness. Inequalities in gametic investment by males and females (anisogamy) often results in different tactics as they attempt to maximize reproductive success (Trivers 1972; Stockley 1997). For example, the use of stored sperm by females can affect male reproductive success through sexual conflict, including cryptic female choice, sperm competition, and post-copulatory sexual selection (Stockley 1997; Jennions and Petrie 2000).

Male reproductive success (RS) is thought to be primarily constrained by mate number (Bateman 1948), but male RS can also be influenced by female qualities such as reproductive frequency, number of offspring, parental investment, and in some species the use of stored sperm, (Darwin 1871; Trivers 1972; Kirkpatrick et al. 1990; Webster et al. 1995). Variation in reproductive qualities of females can result from differences in: 1) resource availability among years, 2) the ability to acquire and utilize resources among females (Stearns 1992), and 3) characteristics of females such as body size and age (Congdon et al. 2003). Compared to males that mate indiscriminately, individuals that have the ability to identify and mate with high-quality females can sire more offspring within and among years.

The characteristics of turtles make them excellent models of less complex reproductive systems (Avise 2001), particularly when genetic and long-term life-history data from the same population are available (Clutton-Brock 1988; Linden and Møller 1989; Clutton-Brock and Vincent 1991). In contrast to mating systems of mammals and birds, less complex systems of poikilothermic vertebrates allows interactions between the sexes that influence reproductive success to be examined in the absence of complex social systems and parental care (Trivers 1972; Clutton-brock 1991; Gross 1996; Avise 2001). Turtles were one of the first vertebrate groups to evolve internal fertilization (Gist et al. 2000); they do not form pair bonds (Wilbur and Morin 1988), and have little or no post-ovulatory parental care (Congdon and Gibbons 1990). Females of many turtle species are intra-seasonally iteroparous (they produce more than one clutch of eggs within a reproductive season), with females of some sea-turtle species laying up to 10 clutches in a year (Eckert 1987). Females often fertilize clutches of eggs with sperm from more than one male (Galbraith et al. 1993; Pearse and Avise 2001b; Pearse et al. 2002; Lee 2008). Female turtles are able to store sperm for longer than a year (Ewing 1943; Gist and Jones 1989) and that allows temporal asynchrony between mating and fertilization (Birkhead and Møller 1993; Shuster and Wade 2003).

Because of the short interval between sequential clutches within a reproductive season , Gist and Congdon (1998) hypothesized that a primary function of stored sperm is to fertilize second clutches. Four studies (Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002; Roques et al. 2006) found that stored sperm was used to fertilize sequential clutches within a reproductive season, suggesting that that use of stored sperm is widespread among turtles. Therefore, variation in reproductive frequency, within and among years, and a propensity for using stored sperm to fertilize sequential clutches, can substantially influence male reproductive success. If males discriminate among female phenotypes that are associated with an individual's tendency to produce single and multiple clutches seasonally, among-individual variation in male reproductive success could increase substantially.

The life-history and ecology of painted turtles (*Chrysemys picta marginata* Agassiz, 1857) have been studied on the University of Michigan’s Edwin S. George Reserve (ESGR) near

Hell, Michigan for 43 of the past 55 years (Sexton 1959; Wilbur 1975; Tinkle et al. 1981; Congdon et al. 2003). A summary of ESGR painted turtle characteristics follows. Males mature at age 4-5 years and females from 6-12 years of age (Congdon et al. 2003), and maximum age of males and females is about 40 and 50 years, respectively. Observations of mating activity of painted turtles on the ESGR are infrequent because it occurs in highly vegetated wetlands in early spring (Sexton 1959). Females allocate the majority of resources to first and second clutch follicles during late summer to early fall in the year prior to egg laying (Congdon and Tinkle 1982). Although females provide no behavioral post-ovulatory parental care, they do provide hatchlings with substantial yolk reserves necessary to sustain juveniles during the early post-hatchling period (Congdon et al. 1983). Some females do not reproduce every year, but the majority oviposit one or two clutches of eggs in terrestrial nests in late May through early July (Tinkle et al. 1981). The shortest interval between first- and second-clutch nests is 10 days, but soft-shelled eggs of second clutches can be detected by palpation six days after the first-clutch nest (J. D. Congdon, unpublished data). Because the size and number of eggs in first and second clutches are similar (Congdon et al. 2003; Harms et al. 2005), producing two clutches of eggs essentially doubles the annual fecundity of females. Temporal and spatial separation of first and second clutch nests reduces the probability that all eggs will be lost (i.e., not putting all eggs in one basket).

Detailed information on the reproductive histories of females from the long-term studies on the ESGR provides an opportunity to interpret the results from the genetic study. Data from the long-term studies include: 1) numbers, body sizes, and ages of most adult males and females in the population, 2) the number of females that reproduced annually, 3) annual reproductive output of individual females (clutch size, clutch frequencies, egg size as egg widths from x-radiographs, and relationships of reproductive variables to body size and age), and 4) the identities of female parents and their offspring. Genotypes of adults and hatchlings allowed us to document the frequency of first and second clutches of intra-seasonally paired clutches being fertilized by the same male (or males) and thereby determine if females used stored sperm to fertilize the second clutch; (Gist and Congdon 1998), whether the same male fertilized sequential clutches between years, and the frequency of multiple paternity. Because both clutch size and egg size increase with body size of females, and egg size and reproductive frequency increases with age (Congdon et al. 2003), older and larger females represent individuals of higher reproductive quality and should be sought after by males.

The primary goals of this study were to test the hypothesis that an important use of stored sperm is to fertilize sequential clutches within a season and quantify age and body size specific reproductive traits of females that may substantially influence male reproductive success.

Materials and Methods

Life history and Population Study

Research was conducted in accordance with the University of Michigan Animal Use and Care Committee (UCUCA #8496). Painted Turtles (*Chrysemys picta marginata*) from East Marsh on the ESGR, were intensively trapped from 1976 – 2007. All individuals were uniquely marked by notching marginal carapace scutes at first capture in aquatic traps, on land, or at drift fences completely surrounding East Marsh or located between wetlands and potential nesting areas. At each capture, the date, individual identification, body size (carapace length, and weight), reproductive condition (e.g., gravid or not gravid) were recorded. Hatchlings and yearlings were assigned “age 1”, and at first capture, the ages of juveniles with distinct growth rings were estimated by assuming one growth ring for each year of life (Gibbons 1976). Thereafter ages of individuals were calculated from the interval between first and each subsequent recapture.

Data on clutch size and reproductive frequency were collected over 26 consecutive years (1983-2006), when East Marsh was completely enclosed by a 1.3 km fence during all nesting seasons. The fence was monitored all days of each nesting season at approximately 20 minute intervals from 0600 h until the end of painted turtle activity in the evening. Almost all females leaving the marsh to nest (with first or second clutches) were captured each year, identified, measured and weighed, and then X-radiographed to determine clutch size and widths of eggs (Gibbons and Greene 1979; Hinton et al. 1997). We associated nests with females by observing them in the act of nesting during extensive searches of nesting areas.

Over the 33 years spanning the entire study at East Marsh (1975-2007), 2,796 individuals were marked, 13,917 recaptures were made, and females were detected with 4003 and 893 first and second clutches, respectively. The total resident adult population at East Marsh between 2001 and 2006 was calculated from catchability data (Congdon and Gibbons 1996) based on all captures and all methods (aquatic traps, on land, or at the fence) over six overlapping three year periods (2000-2002, 2001-2003 2002-2004, 2003-2005, 2004-2006, 2005-2007). We used data from extensive capture and reproductive records from East Marsh (mean = 9.5 years, min-max = 5–24 years) to assign 167 females to the categories of those that had produced ‘one clutch only’ and those that produced ‘second clutches’.

Paired clutches

Within-year pairs of clutches (*N* = 44 pairs; 88 nests) of eggs were obtained from nests of 35 marked females from 2003-2006. Nests were protected in situ in 2003-2004. During 2005 and 2006, eggs were transferred to a common protected natural nesting area within four hours of nest completion. Transferred eggs were incubated in perforated plastic containers to exclude burrowing mammal predation, and were buried approximately 6 cm below the surface of the ground. Different methods of nest protection resulted in similar hatching successes (Chi-square; *P* = 0.4867, df = 32), so samples were combined.

Genetic Samples

A sample of tail tissue (less than 2 mm) was taken from each hatchling and preserved in 95% ethanol until DNA extraction. Approximately 0.1cc of blood was collected from each female parent and was stored in a blood storage buffer (100mM TrisHCL (pH 8.0), 100mM EDTA, 10mM NaCl and 0.5% SDS). DNA was extracted from blood and tissue samples using a Qiagen DNeasy kit (Qiagen), and quantified using fluorimetry.

Hatchlings from all clutches were genotyped at 7 polymorphic microsatellite loci: Cp2, Cp10, Cp3 (Pearse et al. 2001), BTGA2, BTGA3, BTCA7 (Libants et al. 2004), and EB11 (Osentoski et al. 2002). DNA was amplified using the published conditions for polymerase chain reaction (PCR), separated using gel electrophoresis on a 6% polyacrylamide gel, and visualized using an FMBIOII scanner (Hitachi Inc.). Gels were hand-scored independently by two experienced lab personnel and over 10% of PCR products from hatchlings were randomly selected and reanalyzed electrophoretically and rescored independently to minimize scoring errors. Estimates of genetic variability and multi-locus exclusion probability were quantified using the program GERUD 2.0 (Jones 2005) and are based on the equations presented in (Dodds et al. 1996). Analysis of stored sperm and multiple paternity were restricted to clutches with three or more offspring, reducing the sample size to 40 pairs (80 nests) from 31 females. All 44 pairs were used for analyses of hatching success (described below).

Paternal genotype reconstruction

Paternal genotype reconstruction was performed on 40 pairs of nests (80 clutches) using the program GERUD 2.0 (Jones 2005) based on one known (female) parent. We performed three step-wise analyses involving paternal genotype reconstruction: 1) each nest independently, 2) offspring combined for paired clutches within-years, and 3) offspring combined for each female among all years. Reconstructed paternal genotypes of males contributing to nests were also compared visually (Fiumera et al. 2002; Jones et al. 2007) for consistency between first and second clutches that were produced within-years and among years to detect use of stored sperm (below). In cases of multiple paternity, GERUD 2.0 provides potential paternal reconstructions along with estimates of the number of most likely male contributors based on paternal allele counts. Because the female parent was known for all clutches of eggs, we could identify paternal alleles and thus detect multiple paternity when three or more distinct paternal alleles were present. Results from the visual comparison and step-wise GERUD 2.0 analyses were compared for consistency to ensure the most accurate interpretation. Finally, we compared reconstructed paternal genotypes of all contributing males, using the program GENECAP (Wilberg and Dreher 2004), for evidence that any male sired offspring with more than a single female within and among years.

Detecting Repeat Paternity (the use of Stored Sperm)

When paternal genotype reconstructions (GERUD 2.0; Jones 2005) indicated that the same male(s) sired sequential clutches, we identified the second nest as having "repeat paternity". Because of the short interval between first and second clutches within a season, repeat paternity in intra-seasonally paired clutches was considered as evidence of the use of stored sperm to fertilize the second clutch (Gist and Congdon 1998; Pearse et al. 2002). Because there are ample opportunities to re-mate between years, we refer only to repeat paternity among years. However, the male-biased adult sex ratio in the ESGR population (2 males per female) reduces the probability of re-mating with the same male within or between years.

Previous research described the use of stored sperm based on inferences from observations of paternal genotypes being "consistent" or "inconsistent" with respect to the first clutch (Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002). Because of our focus on the influence of female traits on male reproductive success, we expanded the category of "consistent" to include patterns of repeat paternity that differentially influence male reproductive success (Table 1.1). When hatchlings from first and second clutches of an individual female had identical paternal alleles we categorized the condition as "complete" repeat paternity. Complete repeat paternity was then subdivided into "complete type 1" (paternal alleles were consistent with one male) or "complete type 2 (paternal alleles were consistent with more than one male). When the composition of paternal alleles differed between the first and second clutches due to an addition or deletion of paternal alleles, we categorized the condition as "incomplete" repeat paternity. Incomplete repeat paternity was then divided into "inclusive incomplete" (when an allele(s) from a new male was observed only in the second clutch), or "exclusive incomplete" (when an allele(s) from a male siring offspring in the first clutch was not observed in the second clutch). The terminology of inclusive and exclusive incomplete refers to the composition of paternal genotypes present and not whether paternity resulted from re-matings or stored sperm from previous years.

Statistical Analyses

Summary statistics of female attributes and life-history-traits values such as reproductive frequency, age, body size, clutch size, and egg size (width) among females, and demographic parameters such as the number of reproductive females for each year, and correlations between traits were performed using a Spearman's Rank Correlation (SAS 7, 1998)(SAS 1998).

Relationships between female attributes and incidence of multiple paternity were examined using a generalized linear mixed effect, logistic regression model (GLMM), with "paternity" (a binary output with 0 = single paternity and 1= multiple paternity) as the dependent variable (R 2.5 with the lme4 package for mixed models; R Development Core Team, 2007). Since all females contributed at least one pair of nests, and paired clutches for some females were sampled in more than one year, we accounted for structuring in the data by including both female and year as random effects. Fixed effects included female age, female body size (carapace length), clutch sequence (first or second), and clutch size.

When female turtles are isolated from males over several years, use of stored sperm to fertilize subsequent clutches can result in decreased fertility and hatching success (Cuellar 1966; Jun-Yi 1982), via sperm depletion or a decline in sperm viability (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998). We tested whether second clutches had a reduced probability of hatching using a general linear model (GLM) with hatching percent as a dependent variable (rather than a linear mixed effect model) because nests are independent events with respect to hatching percent and the increased power allowed a more accurate test of fixed effects. We also independently tested additional factors proposed to influence hatching success such as female age (Blem et al. 1999), female body size (Ban et al. 2000), and whether a clutch was multiply or singly-sired (Zeh and Zeh 1996; Byrne and Robert 2000; Pearse et al. 2002; Garner and Schmidt 2003; Roques et al. 2006).

Results

Frequency of second clutch production

Over the 24 years after East Marsh was fenced (1983-2006), the number of reproductive females each year averaged 84 (SD = 12.5, min–max = 62-106; Fig. 1.1a) and varied by 46 individuals (43.4%, min-max = 58.5%-100% of the maximum number). The proportion of reproductive females each year that produced second clutches averaged 23% (min–max = 6%-40%; Fig. 1.1b). Variation in numbers of reproductive females among years was reflected in changes in both first and second clutch producing individuals (Fig. 1.2a).

Among 167 females with extensive individual reproductive histories, individual frequencies of second clutch production varied widely (min-max = 0%-50%; Fig. 1.2a). No second clutches were produced by 44 females (26%), whereas 123 females (74%) produced second clutches in some of the years sampled (Fig. 1.2a). Second clutch production averaged 25% (min-max = 7–50%; Fig. 1.2a) for the 123 females that produced second clutches. Carapace length was positively correlated with age within all annual samples (1983-2006) of reproductive females (all Spearman Rho (0.400-0.773), *P*s < 0.006), and among just those females that produced second clutches (all Spearman Rho (0.441-0.852), *P*s < 0.004). In addition, for just second-clutch producing females over all years combined, the frequency of second clutch production was correlated with carapace length (Spearmans *Rho* = 0.235, *P* = 0.01) and age (Spearmans *Rho* = 0.206, *P* = 0.02). The annual percent of second clutches produced in the population was positively correlated with the total number of reproductive females documented each year from 1983-2006 (Spearman's *Rho* = 0.287, *P* < 0.001; Fig. 1.1a,b).

Measures of genetic diversity

The loci Cp2 and Cp10 (Pearse *et al.* 2001) showed evidence of null alleles and were excluded from further analyses. For the remaining five loci, estimates of the measures of genetic variability included the mean number of alleles per locus over five loci (17; min-max = 7-34), mean expected heterozygosity (0.793; min-max = 0.682-0.888), and the expected exclusion probability with one parent known (all loci = 0.994; min-max = 0.437-0.772).

Proportions of the total populations of adult males and females contributing to the paired clutches

Over the four years of genetic sampling (2003-2006), the number of males and females in the East Marsh population averaged 335 (min-max = 321-348) and 180 (min-max = 169–190), respectively (adult sex ratio ~ 2:1). The proportions of high-quality females that were genotyped (those that produced the paired clutches) and males that contributed to hatchlings in those clutches were 6.8% and 9.6% of the estimated total population of each sex, respectively.

Within the sample of hatchlings from 40 pairs of nests, reconstructed male genotypes indicated that no male sired offspring with more than one female that produced two clutches within a reproductive season or among years. However, some males sired offspring with the same female among consecutive years (over two years by two males and three years by one male). In total, 32 males contributed to offspring from 24 females (operational sex ratio = 1.3:1 males to females contributing to paired clutches).

Within-year use of stored sperm

Among 40 paired clutches, 97.5% of the within-year second clutches exhibited evidence that stored sperm was used to fertilize all or a portion of the second clutch of eggs. Among clutch pairs, 77.5% exhibited “complete-type 1” use of stored sperm (i.e., identical paternal alleles indicative of one male in both clutches), 7.5% exhibited “complete-type 2” use of stored sperm (identical paternal alleles indicative of more than one male in both clutches), 7.5% displayed inclusive incomplete use of stored sperm (i.e., some hatchlings had paternal alleles not found in the first clutch), and 5% displayed exclusive incomplete (some hatchlings had paternal alleles not found in the second clutch; Table 1.1). In all cases where overall composition of paternal alleles differed between the first and second clutch, the second clutch showed evidence of incomplete use of stored sperm.

Repeat Paternity Among-years

For seven females, paired clutches were sampled in two consecutive years (14 pairs) and for one female in three consecutive years (3 pairs), allowing 9 among-year comparisons. Overall, 44.4% (4 of 9 among-year comparisons, N = 8 females) of the clutches among years showed evidence of repeat paternity (3 complete type 1, 0 complete type 2, 1 inclusive incomplete, and 0 exclusive incomplete; Table 1.1). One female with clutches sampled over two years, and one with clutches sampled over three consecutive years (3 among-year comparisons;

33.3%), had hatchlings with identical paternal alleles in all clutches. Both clutches of one female in the second year (11.1%) had offspring fertilized by the same male as the previous year and an additional male (evidence of inclusive incomplete use of stored sperm) and one among-year comparison was inconclusive (Table 1.1). Four among-year comparisons (44%) had different compositions of paternal alleles among years (no evidence of repeat paternity). For females where relationships could be conclusively assigned, 50% of among-year comparisons (37.5% of females) showed evidence of repeat paternity among years.

Multiple Paternity

Over 4 years, multiple paternity was evident in 16.3% of all clutches (*N* = 80) and varied among years [22.2% (*N* = 18), 30.0% (*N* = 20), 8.8% (*N* = 34), and 0% (*N* = 8) in 2003, 2004, 2005 and 2006, respectively; Fig. 1.1a]. The proportion of clutches exhibiting multiple paternity was highest in the two years with the fewest reproductive females and lowest in the two years with the most reproductive females. The occurrence of multiple paternity did not differ significantly between first (15%; *N* = 40) and second (17.5%, *N* = 40) clutches (GLMM, *P* = 0.735). However, in 5 out of the 40 pairs (12.5%), single and multiple paternity varied between pairs of clutches, with two pairs having multiple paternity only in the first clutch, and three pairs having multiple paternity only in the second clutch. Multiple paternity was positively, but not significantly correlated with the number of eggs sampled from a clutch (GLMM, *P* = 0.08) and female age (GLMM, *P* = 0.08), but not female body size (GLMM, *P* = 0.52), suggesting there may be an important biological relationship between female age and probability of multiple paternity.

Comparisons of clutch size and egg size in first and second clutches

Over the 24 years (1983-2006), x-radiographs were taken of 312 pairs of clutches of eggs. Mean clutch size was significantly larger (7.2 eggs and 6.2 eggs for first and second clutches respectively, Paired $T = 4.39$, $P < 0.001$), and egg widths (measured from X-radiographs) were slightly and significantly wider (18.1mm and 18.0 mm for first and second clutches respectively; Paired T = 3.40, $P < 0.008$; Table 1.2) in first compared to second clutches. However, min-max of clutch size were identical (3-13 eggs) and egg widths were similar (15.4-20.8mm and 15.9-20.5mm) for first and second clutches respectively (Table 1.2). Over the years in the study for which genetic data are available (2003-2006), the number of offspring genotyped in first and second clutches was not significantly different ($P = 0.256$, df = 87). Hatching success was similar among years (95%, $N = 18$, 90%, $N = 20$, 85%, $N = 40$, and 89.5%, $N = 10$ for 2003, 2004, 2005, and 2006, respectively) and between first and second clutches (90.7 and 86.6% respectively (GLM, $P = 0.285$, df = 87). Hatching success was not associated with female age (GLM, $P = 0.153$, df = 80), body size (GLM, $P = 0.71$, df = 87), or the incidence of multiple paternity (GLM, $P = 0.421$, df = 79).

Discussion

Annual male reproductive success can be affected by among-year variation in 1) total number of reproductive females, 2) the proportion of reproductive females that produce second clutches, and 3) the probability that a given mate will produce a second clutch (Fig. 1.1a,b). That the proportion of second clutches is positively and significantly correlated with the number of reproductive females suggests that resources available during the previous year may be the source of the variation in clutch production (Stearns 1992; Kitaysky et al. 2000). Whereas it may be difficult for males to predict how many females will be reproductive and what proportion will produce two clutches in a given year, resource levels in the present year may allow males to assess the quality of the next reproductive season (e.g., the proportion of second clutches or number of reproductive females).

Among all females, and within the subset of intra-seasonally iteroparous females, age and carapace length (body size) were positively correlated with frequency of second clutch production. Males may be able use phenotypic traits associated with age (e.g., body size) to identify not only females that lay two clutches in a year, but also identify within those females, individuals with high propensity for producing intra-seasonally iteroparous clutches. Additionally, other phenotypic cues may allow males to identify females that lay two clutches. Red and yellow colors on the head, neck, and marginal scutes of adult painted turtles (and in many other species of turtles) may vary in intensity and contrast resulting from variation in carotenoid production, which has been suggested as a reliable indicator of individual quality in other taxa (Kodric–Brown and Brown 1984; Badyaev and Hill 2000; Maan et al. 2006). If males are able to evaluate indicators of female quality to target larger (older) females to increase the probability of mating with an intra-seasonally iteroparous female, the increase in reproductive success would be substantial (even more so if females use stored sperm to fertilize a substantial proportion of second clutches within a year).

Gist and Congdon (1998) hypothesized that an important function of sperm storage in turtles is to facilitate fertilization of sequential clutches within a season because of a short receptive period between clutches. In sea turtles, ovulation of sequential clutches of eggs occurs in less than 36 hr (Licht et al. 1979). Female turtles may not be receptive in the short time between nesting and ovulation of the next clutch of eggs because they must recover physiologically (e.g. from the accumulation of lactic acid during nesting; (Congdon and Gatten 1989; Jessop and Hamann 2004). Further, hormones involved in nesting and mating behavior (e.g., testosterone, estradiol, and progesterone) must readjust to levels suitable for mating (Rostal et al. 1998) because they also appear to provide information about current reproductive state. For example, after gravid females were induced to lay eggs with oxytocin and released back into wetlands, they moved from aquatic to terrestrial nesting areas and performed the entire sequence of nesting behaviors without having eggs to lay (Tucker et al. 1995); J.D. Congdon, unpublished data).

Comparisons of reconstructed paternal genotypes indicated that 97.5% of second clutches of East Marsh females were fertilized with stored sperm [85% complete (combined type I and type II) and 12.5% incomplete (combined exclusive and inclusive)]. The high frequency of stored sperm use found in this study is similar to that in another population of painted turtles (100%) in an open riverine environment (Pearse et al. 2002) and in other species of turtles (Fitzsimmons 1998; Kichler et al. 1999; Roques et al. 2006). We found that sperm from the male that fertilized the first clutch was used to fertilize all or part of the second clutch 77.5% of the time, and two males shared paternity of both clutches 7.5% of the time. In 12.5% of paired clutches, one male was not represented in either the first or the second clutch (incomplete repeated paternity). Incomplete repeated paternity results in a male not siring any offspring in one of the two clutches. Overall, male reproductive success has the potential to dramatically increase without further investment in reproductive activities by mating with females that frequently produce second clutches within a year.

The variation among females in production of second clutches could result in two male tactics: 1) if there is no way to discriminate among females based on their reproductive potential (i.e., clutch frequency), males should mate with as many females as possible (Bateman 1948), or 2) if there are phenotypic cues that allow males to identify females with a high propensity to produce second clutches, males should increase efforts to mate with those females. Bateman (1948) suggested that a primary mechanism for males to increase their reproductive success is through mate number. However, tactic 2 (above) may increase a male's reproductive success while maintaining or reducing the number of matings attempted with different females. Among the ESGR males that successfully mated with an intra-seasonally iteroparous female, none were found to have sired offspring of any other intra-seasonally iteroparous female during the four years of this study. Our results do not support the assumption that mating with as many females as possible is a primary way male painted turtles in this population increase reproductive success.

Perhaps even more striking than the high incidence of repeat paternity within a year is that the same males fertilized all, or a portion of, 44% of pairs of nests among years, and a single male sired all offspring in 6 clutches that one female produced over three years. In European pond turtles, a similar proportion (58%) of clutches fertilized by the same male among years was observed, indicating that among-year use of stored sperm may also be important to male reproductive success (Roques et al. 2006). Because females have more time (and presumably more opportunities) to re-mate among years than within years, it is not possible to exclude re-mating as an explanation for the same male fertilizing paired clutches among years.

The use of stored sperm by ESGR females is the most parsimonious explanation, given a population size of 515 adults and a sex ratio of 1.9 males per female. Our interpretation is that re-mating is not the primary mechanism for fertilizing sequential clutches of a female intra- or inter-seasonally. First, under the assumption of random mating, the probability of a given male mating with a female = (1 male/335 total males), and for mating to occur twice with the same male would be $(1/335)^2 = 8.9 \times 10^{-6}$. Second, between reproductive seasons the majority of females almost certainly encounter many potential mates. Third, the use of stored sperm reduces the necessity for spatial and temporal synchrony of matings with female receptivity (Birkhead and Møller 1993; Shuster and Wade 2003), which would allow for more time (and presumably opportunity) to mate with more females. Finally, there is no evidence of pair bonding in turtles (Galbraith 1993; Pearse and Avise 2001a; Roques et al. 2006).

If stored sperm is the mechanism that explains the repeat paternity among years, then the question becomes: Why do females use stored sperm when males are plentiful and frequently encountered? Regardless of whether repeat paternity is the result of stored sperm or re-mating, the findings suggest that once a male is successful with a female, he has a high probability of continuing to be successful with that female among years. Either way, the results are not consistent with the general perception that the number of mates is the only substantial determinant of male reproductive success.

Clutch sizes and hatching success were similar in first and second clutches. Female turtles are able to store sperm over several years (Ewing 1943) and although declines in fertility have been documented in turtles (Palmer et al. 1998), the decline in fertility was observed among years, not within a season. The high probability that females use stored sperm to fertilize the second clutch and that the hatching success of first and second clutches were similar, indicates that mating with intra-seasonally iteroparous females has the potential to substantially increase male reproductive success.

Two major influences on male reproductive success of painted turtles on the ESGR appear to be the potential that the females have for producing second clutches and the propensity for females to use stored sperm to fertilize sequential clutches within and among years. If males have no way of discriminating among females, then the intensity of courtship by males and the cost and benefits of courting or defending any female in the population should be equal. However, if males can recognize high quality females (those that produce and use stored sperm to fertilize multiple clutches within a year), the costs may increase since more males would be attempting to mate with them and that could result in reducing a male's ability to mate with other females. Even with increased costs, the benefits to males would apparently remain high for those mating with females that are intra-seasonally iteroparous.

The large proportions of sequential clutches within a year that are fertilized with stored sperm in different species (Fitzsimmons 1998; Kichler et al. 1999; Pearse et al. 2002; Roques et al. 2006); this study) suggest that use of stored sperm within a year is widespread among turtles. In many species of sea turtles, females lay 2-10 clutches of eggs in a single season (van Buskirk and Crowder 1994). If repeat paternity is observed among clutches within a year (e.g. Fitzsimmons 1998), then the benefits to male reproductive success could be great. However, the inter-annual nesting intervals can extend 5 years or more (Limpus et al. 1994), a period that may be too long for stored sperm use among-years. Additionally, with clutch sizes much larger (many species over 100 eggs per clutch; Buskirk and Crowder 1994) than those observed in painted turtles (average of approximately 7 eggs per clutch), the issue of sperm depletion within and among years must also be considered in sea turtles. Females in other taxa (including birds, reptiles, mammals, and insects) are also known to store sperm (Birkhead and Møller 1993; Birkhead 1998), and several species are intra-seasonally iteroparous (Verhulst et al. 1997). If frequent use of stored sperm to fertilize offspring in sequential reproductive bouts (within or between years) occurs in these groups, it represents a potentially important component of male reproductive success.

APPENDIX 1

Table 1.1. Demonstration of the use of inferred paternal alleles at a polymorphic locus to distinguish among four categories of repeat paternity (stored sperm use) that can have direct influence on male reproductive success (RS) of painted turtles (*Chrysemys picta marginata*).

	Paternal Alleles		**Observed Incidence**		
Categories of Repeat Paternity	**First Clutch**	**Second Clutch**	**Within Years *N* = 40 pairs**	**Among Years *N* = 9 comparisons***	**Implication of repeat paternity for Male RS**
Complete: Type 1	A B	A B	77.50% (*N*=31)	33.30% (*N*=3)	One male attains 100% paternity of two clutches
Complete: Type 2	A B C	A B C	7.50% (*N*=3)	0.00%	Two males attain partial paternity of both clutches
Inclusive Incomplete	A B	A B C	7.50% (*N*=3)	11.10% (*N*=1)	Male 1 attains complete paternity of clutch 1 and partial paternity of clutch 2 Male 2 attains partial paternity of clutch 2
Exclusive Incomplete	A B C	A B	5.00% (*N*=2)	0.00%	Male 1 attains partial paternity of clutch 1 and 100% paternity of clutch 2 Male 2 attains partial paternity of clutch 1 and 0% paternity of clutch 2
Inconclusive	A B C	B D	2.50% (*N*=1)	11.10% (*N*=1)	Unable to determine without paternity analysis
No Evidence	A B C	D E F	0.00%	44.40% (*N*=4)	Males unable to capitalize on repeat paternity

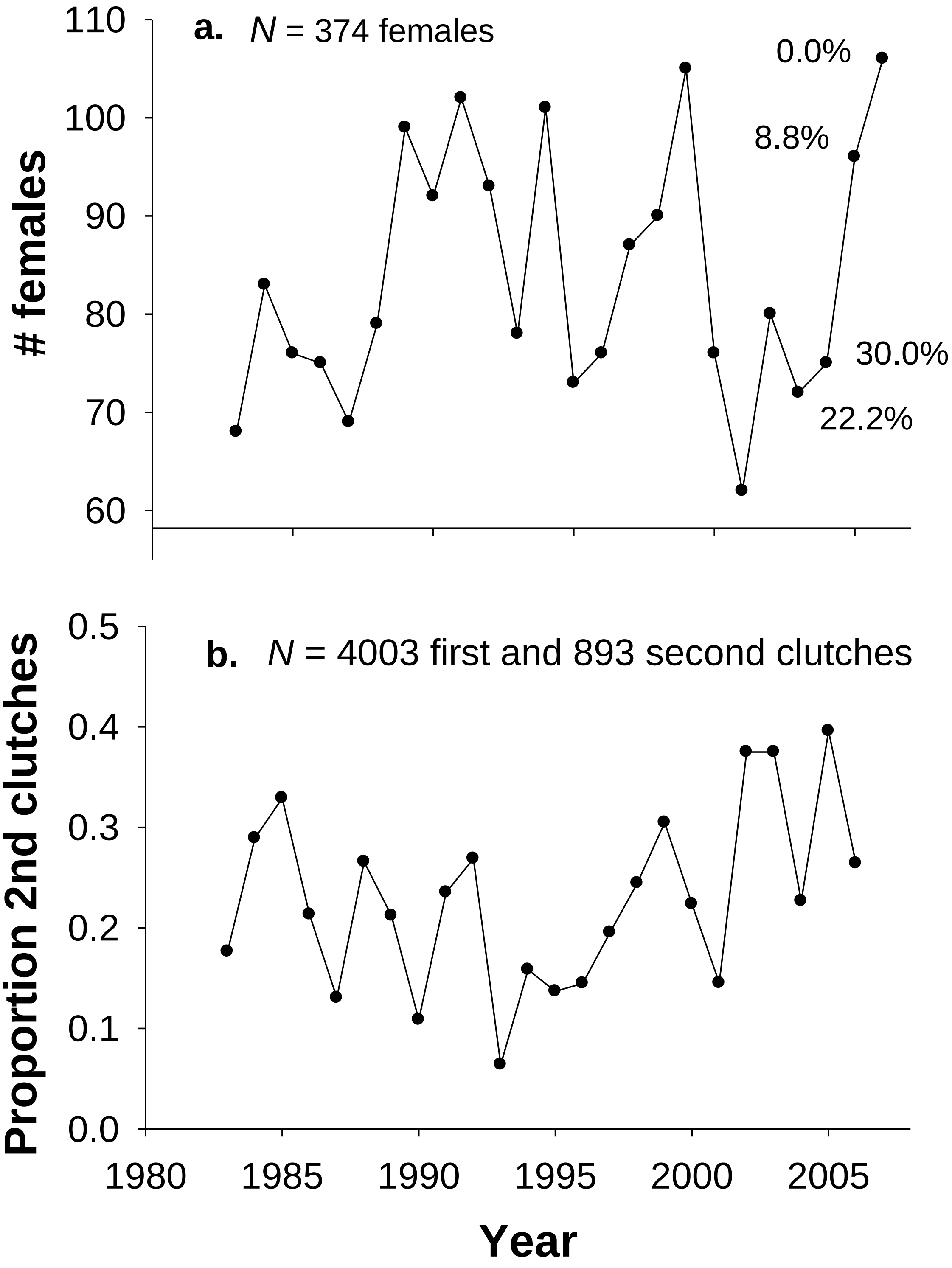
*Nine among-year comparisons with one female sampled in three years (2 comparisons)

Table 1.2. Clutch size and egg widths (from x-radiographs) of 44 female painted turtles (*Chrysemys picta marginata*) that did not produce any second clutches and 123 that did produce at least one second clutches over sampling periods of 5-21 years (data = mean, standard deviation, (minimum-maximum).

Female Category	*N* Clutches	Intra-seasonal clutch sequence	Clutch size	Egg width (mm)
No second clutches	263	First only	6.7.0; 1.53 (2-11)	17.6, 0.87 (15.1-19.5)
Second clutches	312 Pairs	First	7.2, 1.46 (2-13)	17.9, 0.94 (15.4-20.8)
		Second	6.2 (3-13)	18.0, 0.85 (15.8-20.5)

Fig. 1.1. a) The total number of reproductive painted turtle (*Chrysemys picta marginata*) females captured each year at East Marsh (numbers with the last four years are the percent of clutches exhibiting evidence of multiple paternity in years for which genetic data were available), and b) the proportion of second clutches produced at East Marsh each year from1983-2006.

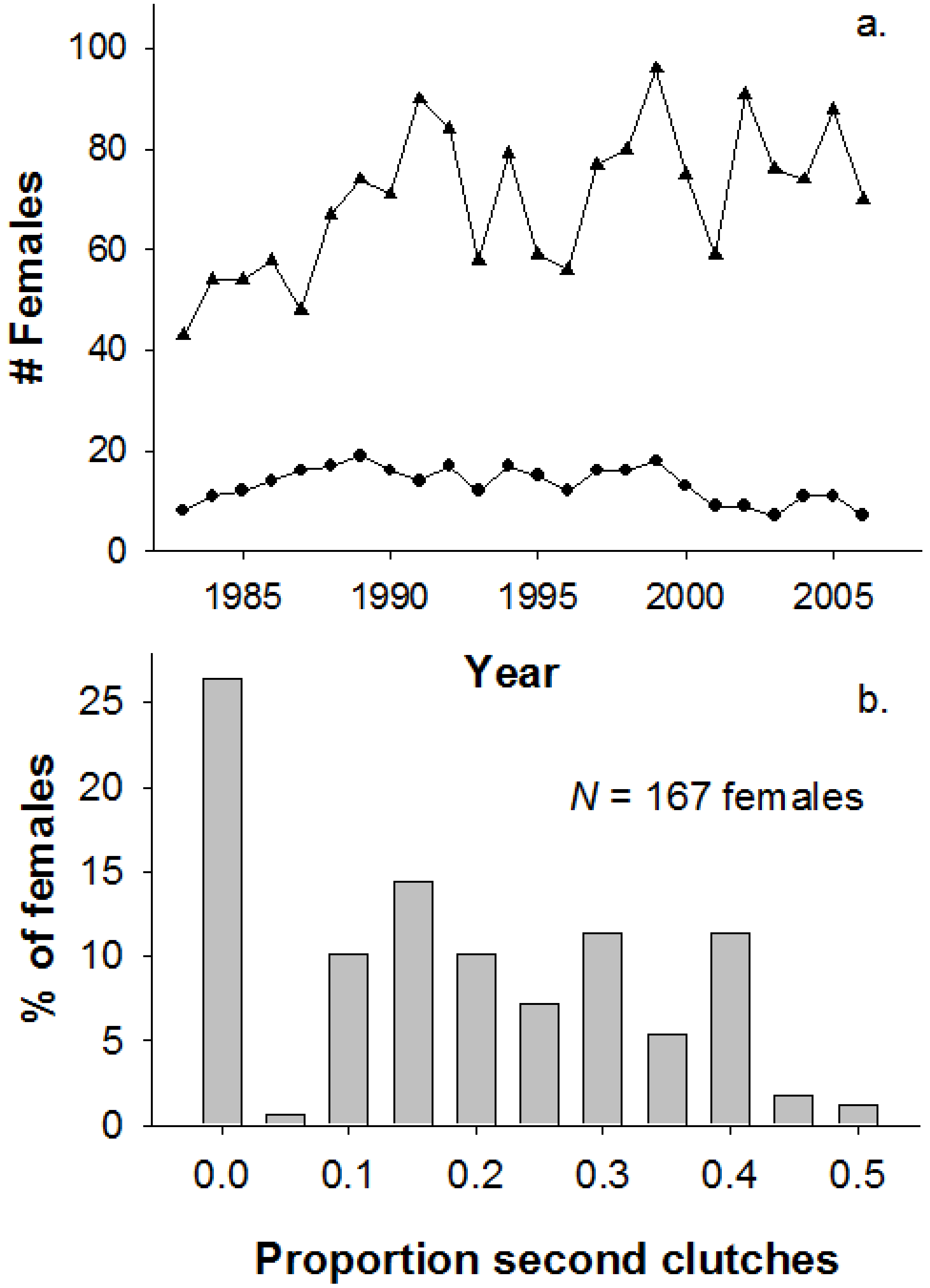
Fig 1.1 (cont'd)



a. N = 374 females
females
110
100
90
80
70
60
0.0%
8.8%
30.0%
22.2%
b. N = 4003 first and 893 second clutches
Proportion 2nd clutches
0.5
0.4
0.3
0.2
0.1
0.0
1980
1985
1990
1995
2000
2005
Year

Fig. 1.2. a) The numbers of painted turtle (*Chrysemys picta marginata*) females with reproductive records spanning 5–24 years) that produced first clutches only (circles), and those that produced second clutches (triangles) each year (1983-2006), and (b) percent of females producing second clutches (on the x-axis, 0.0 indicates females that did not produce any second clutches).

Fig 1.2 (cont'd)


a.
Females
0
20
40
60
80
100
1985
1990
1995
2000
2005
Year
b.
N = 167 females
% of females
0
5
10
15
20
25
0.0
0.1
0.2
0.3
0.4
0.5
Proportion second clutches

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CHAPTER 2: FEMALE QUALITY AFFECTS MALE PAINTED TURTLE (CHRYSEMYS PICTA MARGINATA) REPRODUCTIVE SUCCESS

ABSTRACT

Male reproductive success (RS) is often associated with mate number. However female reproductive qualities (e.g., clutch size, egg size, and clutch frequency) and propensity to use stored sperm to fertilize sequential clutches can also contribute to male RS. Life history data on Painted turtles (*Chrysemys picta marginata*) collected from 1983-2007 were used to quantify variation in reproductive quality of females. Genetic determinations of paternity estimated from 155 nests from 59 females during 2003-2006 were used to quantify the number of offspring sired by each male. Mate quality (primarily intra- and inter-annual clutch frequency) was the major determinant of male RS rather than mate number. The number of offspring sired was influenced by repeat paternity, resulting from rematings or female use of stored sperm, that occurred in > 95% and 38% of clutches produced within or among years, respectively. Older females produced clutches of eggs both within season and among years more frequently than did young individuals. The incidence of multiple paternity was also significantly higher in clutches of older females, particularly in years when the total numbers of reproductive females were low. However, males sired offspring with only one female within years (1.05 females) and over four years (1.09 females). Results suggest that mating with more than a single female is difficult or costly; however, factors such as prolonged courtship or mate guarding have not been reported in turtles. The skewed adult sex ratio (2.3 males/female) may contribute to costs associated with courtship and successful mating of males and females.

Introduction

A combination of ecological, demographic and genetic data allow the relative importance of factors influencing male reproductive success (RS) to be indentified (Rowell and Servedio 2009; Salehialavi et al. 2011). Although male RS is often associated with mate number (Bateman 1948; Clutton-Brock and Vincent 1991; Andersson 1994), RS of males may also be influenced when females vary in quality (Andersson 1994; Owens and Thompson 1994; Servedio and Lande 2006).

The relative costs and benefits of male mating tactics can be difficult to discern in species with complex social systems. Therefore we examined the relative influence of mate number and mate quality has on male RS in the relatively simple social system of Painted turtles (*Chrysemys picta*). Painted turtles do not form pair bonds, have relatively simple courtship behaviors, and provide no post-ovulatory parental care; however, females frequently use stored sperm to fertilize second clutches (Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002; McGuire et al. 2011). Reproductive qualities of female Painted turtles include clutch size, egg size, and reproductive frequency (number of clutches produced within and among years) and all increase with female body size and age in the study population (Congdon and Gibbons 1987; Congdon et al. 2003). Approximately one third of females in study population produce only one clutch of eggs per year, two thirds produce more than one clutch within a season (intra-annual iteroparity), and some females do not reproduce each year (McGuire et al. 2011).

Although painted turtles live in resource rich wetlands, per capita resource availability may be limited due to resource harvest and processing costs (Congdon 1989). If resources are limited, production of second clutches in a given year may result in longer annual intervals between reproductive bouts and that would reduce differences in reproductive output between females that produce only one versus females that produce two clutches annually. Regardless of potential constraints on second clutch production, variation in female qualities will influence the RS of males, and if males can access female quality, selection should favor males that do so.

A study of the life history, demography, and reproductive ecology of Painted turtles on the Edwin S. George Reserve (ESGR) near Hell, MI was conducted each year from 1975-2007. We used detailed information on age- and size-specific variation among individuals of both sexes, and long-term data on clutch size, egg size, and inter-clutch intervals of individual females to document variation in female reproductive qualities.

Long term life history and population size data were combined with genetics data on repeat paternity, multiple paternity, mate number to support interpretations and inferences about the mating tactics and male RS. Genetic data establishing paternity of offspring from nests were collected over 4 years to quantify how variation in the number and reproductive quality of females influences male RS within and among years.

The goals of the study were to test the following predictions: 1) if resources are limiting, the length of inter-clutch intervals will be longer for females producing second clutches than for those producing a single clutch, 2) mate number will be a substantial component of male RS; 3) female quality will be a substantial component of male RS; and 4) if males can assess reproductive quality of females then individuals with high qualities (e.g., larger or older females that have larger clutch sizes and shorter inter-clutch intervals) will have a higher incidence of multiple paternity.

Materials and Methods

Field sample collection

Life history and genetic data were restricted to a single permanent wetland on the ESGR (East Marsh) that was completely encircled by a 1.3 km drift fence from 1983-2006. The fence was located between the wetland and nesting areas and was walked at approximately 20 min intervals from 0600 h until the end of Painted turtle activity in the evening during all days of the nesting season. Because nesting almost always takes more than an hour to complete, the monitoring frequency on the fence was frequent enough to observe any females attempting to construct a nest near the fence. Therefore, almost all females leaving the marsh to nest each year (with first and second clutches) were captured and identified (Gibbons and Greene 1979; Hinton et al. 1997).

During 15 of the years from 1976-2007, Painted turtles from East Marsh were intensively trapped (mean aquatic captures per year 360, min–max = 219-578, SD = 105.06). During the other years the study was restricted to the nesting season and trapping was less intense. At first capture (in aquatic traps, dip nets, by hand, on the fence, or on land), all individuals were uniquely marked by notching marginal carapace scutes. At each capture the date, location, sex, and body size (carapace length) were recorded. Just prior to and during the nesting season, all females all females on land were X-radiographed to determine clutch size and widths of eggs. At first capture the ages of juveniles with distinct growth rings were estimated by assuming one growth ring for each year of life (Gibbons 1976). Hatchlings and yearlings were assigned “age 1”. Ages of individuals were then calculated from the interval between the first capture (where age was assigned) and each subsequent recapture.

Data on the number of reproductive females, their body sizes and ages, clutch sizes, egg widths, were collected for each of 24 consecutive nesting seasons (1983-2006), the period that

East Marsh was enclosed within a drift fence. Data from extensive captures and reproductive records of 167 East Marsh females (mean recapture intervals = 12.0, min-max = 5-33 years, SD = 6.45) were used to assign individual probabilities of second-clutch production.

Offspring were associated with mothers by observing females in the act of nesting during frequent searches of nesting areas. Post-nesting females returning to the fence without eggs were recorded as non-gravid. Assignment of a reproductive event as a first or second clutch was based on recapture histories when gravid females exited the wetland 10 or more days after their first clutch and from differences in the number or position of eggs in sequential X-radiographs within a nesting season. Because a few females did not return the East Marsh after nesting, clutches of any individual caught in a nesting area > 15 days post first clutch (supported by X-radiograph data when available) were also assigned as a second clutch.

Hatchling sample collection

From 2003-2006 a total of 155 clutches of eggs and 1054 hatchlings obtained from 59 marked females were used for genetic analyses (see below). Nests were protected *in situ* in 2003-2004, and eggs were transferred to a common protected natural nesting area within four hours of nest completion during 2005 and 2006. Transferred eggs were incubated in perforated plastic containers to exclude burrowing mammal predators, and were buried approximately 6 cm below the surface of the ground.

Genetic data collection

Approximately 1cc of blood was taken from adults and stored at room temperature in a buffered solution (100mM TrisHCL (pH 8.0), 100 mM EDTA, 10 mM NaCl and 0.5% SDS) until DNA was extracted. A < 2 mm tail tip sample was taken from all hatchlings and preserved in 95% EtOH and blood samples were obtained from the caudle vein of adults and preserved in blood storage buffer. Both sample types were stored at ambient temperature until they were moved to refrigeration (4°C). DNA was extracted from blood from adults (N = 535) and tissue from hatchlings (N = 1054) using a Qiagen DNeasy kit (Qiagen Inc, Valencia, CA) and quantified using fluorimetry.

All samples were genotyped at 7 microsatellite loci (Cp2, Cp3, Cp10, (Pearse et al. 2001a), BTGA2, BTGA3, BTCA7, (Libants et al. 2004) and EB11 (Osentoski et al. 2002). Amplification was performed using polymerase chain reaction (PCR) using the published conditions and separated using gel electrophoresis on a 6% polyacrylamide gel. Products were visualized using an FMBIOII scanner (Hitachi Inc., Tokyo, Japan). All gels were independently scored by a minimum of two experienced laboratory personnel. To estimate error, and ensure genotyping consistency among samples, 10% of adults were randomly selected and re-genotyped for all loci. Estimates of expected heterozygosity, number of alleles, the exclusion probability with one parent known, and tests for Hardy-Weinberg Equilibrium were performed using the program CERVUS 3.1.0 (Kalinowski et al. 2007).

Paternity Assignment

Some commonly used parentage programs, such as CERVUS 3.0.1 (Kalinowski et al. 2007) or COLONY (Wang 2004) can over-estimate the number of contributing sires (Fiumera et al. 2002; Jones et al. 2007), particularly when offspring are non-independent. Such is the case when each offspring within a clutch shares the same known maternal parent. As a result, treating each offspring as an independent event fails to incorporate prior information available in the progeny arrays, and can lead to over-estimation of the number of contributing sires (Jones et al. 2007).

Because the mothers of Painted turtle offspring produced during the study were known based on direct observation while constructing nests and offspring are grouped within a nest, we used a two-tiered approach to assign a male to each offspring. We first assigned male(s) to a clutch using the program NEST (Jones et al. 2007). We then assigned males to individual offspring without the clutch grouping using program CERVUS 3.0.1 (Kalinowski et al. 2007). We assigned a male to each offspring when the male(s) assigned by the NEST program also were determined to have high probability of paternity based on a positive LOD score and with no sire-offspring genotype mismatches as determined by program CERVUS. Joint assignment of males to offspring by both programs reduces over-estimation bias. We also reconstructed male genotypes from offspring genotypes for each clutch using program GERUD 2.0 (Jones 2005). Reconstructed male genotypes were compared to the genotypes of candidate ESGR males using the program GENECAP (Wilberg and Dreher 2004) that allows reconstruction and comparisons of multi-locus genotypes from males. GERUD 2.0 is highly accurate at assessing the number of sires for a clutch when there are fewer than 6 sires (Sefc and Koblmuller 2008). The paternal assignments from program NEST were compared to the assignments from GENECAP and GERUD 2.0 along with visual assignments using the method described by (Fiumera et al. 2002) to ensure consistency in paternal assignment. Repeat paternity was identified when the same male was observed siring offspring with the same female in successive clutches within or among years.

Statistical Analyses

Female reproductive quality - We tested whether female age was predictive of reproductive frequency using mixed model analysis (GLMM). Reproductive intervals were estimated as the number of years between successive reproductions (two clutches within a year were assigned an interval of 0). Inter-nest intervals of > 4 yrs were removed from analyses because durations of 5 or more years were considered cases where pre-nesting movements occurred prior to monitoring the fence or were due to injury of disease.

Because extensive reproductive histories were available for each of 167 females and all females reproduced a number of times over the period 1983-2006, we included females as a random effect with a random slope and intercept (Schielzeth and Forstmeier 2009). We assigned female age, body size, and reproductive frequency as fixed effects. Of the 167 females with extensive reproductive histories spanning 5-30 years, 44 produced a maximum of one clutch per year and 123 produced a maximum of two clutches a year. We examined the variation among females for relationships within season and among year reproductive frequencies. Specifically, we tested whether the production of a second clutch (at year t) increased the time until the next clutch was produced (during year t+1) using a binomial parameter (0,1) indicating whether the preceding reproductive event involved was characterized by production of one or two clutches.

Male reproductive success - We quantified whether male attributes were significantly associated with offspring numbers sired using linear mixed models (GLMM); restricting analysis to only successful males. We included female as a random effect and tested the fixed effects of male age and body size, total number of mates, total number of clutches sired per year, number of clutches sired in all years, and multiple paternity of clutches sired on male RS (defined as the total number of offspring sired).

We also tested whether body size and ages of males and females and clutch size of females were associated with incidence of multiple paternity. To reduce structuring in the data set, female and male parameters were run in separate models. For example, associations between the occurrence of multiple paternity and female attributes were tested in models where each clutch was represented once. Male attributes associated with multiple paternity were tested in models where a clutch may be represented as many as two times (once for each male).

Evidence for assortative mating- We tested for evidence of assortative mating as a function of ages and sizes of males and females of mating pairs using two independent tests. First, we used a mixed model to determine whether male age and size was predicted by the mated female's age and size. We included female as a random effect (intercept) in all models. Second, we tested whether ages or sizes of mated pairs deviated from random pairs of males and females by generating random mating pairs from a pool of all adult females and males sampled. Because not all females reproduce in every year, and some females reproduce in more than one years, we randomly sampled (with replacement) males and females to produce 155 mating pairs to have a similar sample size as our genetic data. For each mating pair, we calculated ratios of female age/size to male age/size (e.g., female age divided by male age for the mating pair) as described by Rowe et al. (2007). Frequency distributions of ratios for the randomly generated mating pairs were then compared with the actual mating pairs using a G-test. All statistical analyses were implemented in the statistical software R (R Development Team 2006).

Results

Female Reproductive Qualities.

Over the 24 years that East Marsh was fenced (1983-2006), an average of 91.5 females reproduced each year (Fig. 2.1a; min–max = 55-102; *SD* = 16.04), and on average, 26.0% (min – max 13.0% – 45.0%, *SD* = 0.89) of nesting females produced second clutches each year. Because female body sizes and ages are positively correlated (Fig. 2.1b) and both are positively correlated with reproductive qualities (Congdon and Gibbons 1987; Congdon et al. 2003), we examined whether body size and ages varied among years with different numbers of females. The ranks of carapace lengths, ages, and number of reproductive females each year were not correlated (Spearman Rho = 0.351, *P* = 0.09); Spearman Rho = 0.707, *P* = 0.74, respectively).

Clutch size and egg widths of first clutches averaged 6.9 (min-max = 3-12, *SD* = 1.48, *N* = 1479) and 17.6 mm (min-max = 13.6-22.0, *SD* = 1.03, *N* = 1607), respectively and for second clutches averaged 6.7 (min-max = 3-13, *SD* = 1.55, *N* = 364) and 17.8 mm (min-max = 15.6-17.9, *SD* = 1.03 *N* = 359), respectively. Clutch size and egg width were positively correlated with body size of females (Fig. 2.2a,b). The frequency of second-clutch production increased from 10% in primiparous females to 40% in the oldest females (Fig. 2.2c).

Among 167 females with extensive reproductive histories spanning 5-33 years, 44 (26%) produced only one clutch in any year and 123 produced second clutches in 7-50% of years monitored. The average frequency of second clutch production of all 167 females was 20% (min-max = 0% - 50%, *SD* = 0.14). Among just those 123 females that produced second clutches, second clutch production averaged 25% (min-max = 7–50%). Females with the highest second clutch production rates had lower inter-year reproductive intervals (GLMM, *t* = -3.50, *P* <0.001), and older individuals had shorter reproductive intervals than did younger females (GLMM, *t* = -2.141, *P* < 0.05). Among-year reproductive intervals of females that only produced first clutches were longer (mean = 1.39 years, *SD* = 0.73, min - max = 1-4, than the among year intervals females that produced two clutches per year (mean = 1.13 years, *SD* = 0.41, min-max 1-4; GLMM; *t* = -3.96, *P* < 0.001).

From 1999-2006 (the years that genetic samples were taken from adults), a total of 216 gravid females were captured at East marsh, and the number of females that reproduced each year averaged 89 (min – max 70 – 106, *SD* = 13.13). In 1999, 100 reproductive females were captured, and 116 different reproductive females were captured over the next 7 years. The range of body sizes of the 116 females was similar to the females in the 1999 sample (Fig. 2.3a). A mean of 16.6 (min-max = 11-28; *SD* = 5.80) new females were captured in each of the following 7 years and 42% (12% - 65%) of individuals were 6-11 years old (the ages of primiparous females that generally have low reproductive qualities). Compared to the distribution of females ages in 1999 (Fig. 2.3b), the distribution of ages of new individuals captured from 2000-2006 was biased toward young females (Fig. 2.3c). The proportions of new females < 12 years of age captured during the years genetic samples were taken from hatchlings (2003-2006), were 45, 43%, 43%, and 65%, respectively.

Age and Size Distributions of Females

Body sizes (carapace length) of reproductive females with nests sampled to determine paternity (*N* = 59 females) from 2003-2006 were not different from females captured from 1983-2006 (Fig. 2.4b; Chi-square, $X^2 = 2.85$, df = 6, *P* = 0.827). Age distributions from 2003-2006 were normally distributed (Fig. 2.5a), but differed significantly from ages of reproductive females captured from 1983-2006 (Fig. 2.5b; Chi-square, $X^2 = 48.81$, df = 6, *P* < 0.001). The size and age distributions of females in years with low numbers of reproductive females (2003 and 2004; Fig. 2.4c,d and Fig. 2.5c,d) were not different from the distributions of sizes and ages of females in years with high numbers of reproductive females (2005 and 2006; Fig. 2.4e,f and Fig. 2.5e,f; $X^2 = 3.29$, df = 6, $P = 0.772$, age, $X^2 = 7$, df = 6, $P = 0.321$).

Genetic data and patterns of variation in multiple paternity

All loci were polymorphic and in Hardy-Weinberg Equilibrium. Estimates of genetic variability included the mean number of alleles per locus over the five loci (mean = 17; min-max across loci = 7 - 34), mean expected heterozygosity (0.793; min - max among loci = 0.682-0.888), and the expected paternal exclusion probability with one parent known was 0.998. Empirically estimated multi-locus error rate was less than 1%. No evidence of gametic disequilibrium was detected ($P > 0.05$).

Of the 155 nests sampled to determine paternity, males were assigned to offspring in 108 nests (70%). Concordance in paternity assignment among programs (NEST, CERVUS, GERUD with GENECAP) was 94%. Nests where offspring were assigned inconsistently among programs were excluded from further analyses. Comparisons between paternity assignment using the multiple-program approach to the "most-likely male" approach from CERVUS resulted in 24.7 % of offspring assigned to different males, and in all cases would have resulted in an overestimation of the number of contributing sires (as well as multiple paternity).

Multiple paternity was observed in 14.1% of clutches of eggs sampled and the proportional occurrence varied among years (30.0, 12.5, 14.3 and 6.1% for 2003-2006, respectively). The incidence of multiple paternity in clutches of females that produced second clutches was similar to clutches of females that only reproduced once in a year (13.5% versus 13.8%; $X^2 = 0.048$, df = 1, $P = 0.83$). Assuming that all males remain reproductive each year, variation in the total number of reproductive females will cause the operational sex ratio in East Marsh to change over the four years that multiple paternity was determined (Fig. 2.1a). Therefore, we tested whether factors associated with incidence of multiple paternity differed in years with high (2005, 2006) or low (2003, 2004) numbers of reproductive females. The proportion of clutches from second-clutch producing females that were multiply sired was different between the two years with high and two years with low numbers of reproductive females ($X^2 = 292.55$, df = 11, $P < 0.001$). In years with low numbers of reproductive females, offspring in one or both clutches of females producing second clutches were significantly more likely to be sired by >1 male (higher incidence of multiple paternity) than clutches of females producing single clutches (20.9 vs. 12.5%, respectively). In years when the number of reproductive females was high, the incidence of multiple paternity was 9.2% and 14.3% in females producing only single clutches and those producing second clutches, respectively, but the differences were not significant.

Overall, clutch size and female body size (carapace length) were not significant predictors of multiple paternity (GLMM, clutch size, $Z = -0.316$, $P = 0.752$, body size = $Z = -0.228$, $P = 0.819$). Female age was a significant predictor of multiple paternity over all years (GLMM; age, $Z = 2.013$, $P = 0.044$), and in years with low numbers of reproductive females, (GLMM; $Z = 2.652$, $P = 0.008$). Female age was not significant in years with high numbers reproductive females (GLMM; $Z = 0.305$, $P = 0.760$). No other factors tested were significantly associated with incidence of multiple paternity (GLMM; $P > 0.10$). The incidence of multiple paternity was not different in the next clutch for females reproducing and those skipping reproduction in a given year ($X^2 = 0.142$, df = 1, $P = 0.706$).

Variation in Repeat paternity

The same male sired offspring with the same female between years (repeat paternity) in 38% of nests, including inter-nest intervals of up to 4 years (Fig. 2.6). There was no significant relationships between repeat paternity and the age or size of females (GLMM; age, $Z = 0.043$; $P = 0.966$; size, $Z = 0.792$, $P = 0.429$) or males (GLMM; age, $Z = 1.037$; $P = 0.300$, size, $Z = 1.032$; $P = 0.302$). Repeat paternity was not predicted by multiple paternity (GLMM, $P = 0.996$).

Male reproductive success

The number of offspring sired varied among successful males each year (mean = 7.11, min - max = 1 - 19 offspring, *SD* = 3.99; Table 2.1) and among the four years of study (mean = 8.83, min - max = 1 - 33 offspring, *SD* = 6.28). Male RS (number of offspring sired from 2003-2006) was not predicted by the number of mates (GLMM, $t = 1.465$; $P = 0.144$). The average number of mates per year was 1.05 (*SD* = 0.22), and the average number of mates over the four year period (2003-2006) was 1.09 (*SD* =0.30). No males sired offspring with more than one female in any year except 2005 (mean = 1.15 mates).

The number of clutches sired was a significant predictor of male RS (GLMM, $t = 10.114$; $P < 0.001$). The number of clutches sired averaged 1.4 per year (*SD* = 0.57) and 1.7 (*SD* = 0.87) over the 4 years of study. Therefore increases in the number of offspring sired primarily resulted from repeat paternity (use of stored sperm or remating with the same female). We also tested the influence of the number of clutches on male RS resulting from only among-year variation in the number of clutches by randomly removing either the first or second clutch of each pair. When the total number of offspring and number of clutches sired were recalculated, the number of clutches sired was still a significant predictor of male RS (GLMM, $t = 11.708$, $P < 0.001$). Therefore, repeat paternity among years also has a substantial influence on male RS. Age and size distributions of successful males did not differ from all sampled males (Fig. 2.7; age, $X^2 = 6.973$, df = 8, $P = 0.540$; size, $X^2 = 19.226$, df = 13, $P = 0.116$). Among successful males, the total number of offspring sired was not associated with male body size or age (GLMM; size: $t = 1.698$; $P = 0.095$), GLMM; age: $t = -0.936$, $P = 0.361$), but was marginally negatively influenced by multiple paternity (GLMM; $t = -1.89$; $P = 0.06$).

Evidence for Assortative mating

Frequency distributions of sampled mating pairs differed substantially from random mating with respect to age ($G = 20.17$, df = 7, $P = 0.005$; $X^2 = 13.71$, df = 7, $P = 0.056$) but not body size ($G = 1.39$, df = 7, $P = 0.986$; $X^2 = 0.73$, $P = 0.994$). The ages of females were correlated with the ages of male mates (GLMM, $t = 2.67$, $P = 0.01$), whereas body sizes of females and males was not (GLMM, $t = 1.23$, $P = 0.20$).

Discussion

Despite the almost stable population size of Painted turtles on the ESGR, the number of gravid females captured varied annually 55-102 (mean = 91.5). From 1999-2006, the ages of the majority of different reproductive females captured after 1999 were ages 6-12 years (the ages of primiparous females) whereas the others were presumably those that had skipped reproduction. During the last 3 years of the study (200-2006), we trapped East Marsh extensively and made 25 captures of 22 females that had not been captured on land during the nesting season (i.e., did not reproduce that year); an average of 7% of females captured.

Approximately 26% of reproductive females were not observed to produce any second clutches over intervals of 5-32 years whereas 74% produced clutches in 7-50% of all years. Individuals of both groups skipped reproduction in some years. The minimal interval between within season clutches is approximately 10 days and among year intervals exceed two years. Over the 24 years that East Marsh was fenced (1983-2006), and during the period of genetics study (1999-2006) the average number of females that reproduced each year were similar (means = 91.5 and 86.6 females, respectively). The range of variation in numbers of reproductive females and the sample of females that do not produce second clutches in any years was surprising since females live in a resource rich wetland (Fiala and Congdon 1983) and females use body lipid stores to support reproduction (Congdon and Tinkle 1982). However, there are other short-lived (*Deirochelys reticularia,* Gibbons 1969) and long-lived (*Chelydra serpentina,* Obbard 1983; *Emydoidea blandingii,* Congdon et al. 1993) adult female turtles that do not reproduce every year. That some females do not reproduce each year, while some females can produce two clutches in one year, represents a source of variation that can have direct benefits to male RS.

Because clutch size and egg size of first and second clutches are similar, females that produce second clutches allocate approximately two times the resources to reproduction in a year than do females that produce one clutch. If resource limitation occurs in the relatively resource rich environment of a wetland, we expected that it would be expressed as a longer inter-annual reproductive interval (i.e., narrowing the difference in reproductive quality). However, the inter-annual intervals between reproductive events were similar in females that produce only one clutch per year and those that produce second clutches (i.e., inter-annual reproductive intervals do not influence differences in reproductive quality). In addition, among all females, older females have shorter intervals between reproductive events and among just the females that produce two clutches, older females produce paired clutches at higher frequencies (McGuire et al. 2011).

When females vary substantially in reproductive quality, males should evolve to prefer traits that are reliable indicators of the direct benefits (Gwynne 1981). Males demonstrate preferences for reliable indictors of female quality in a variety of taxa (Amundsen 2000; Clutton-Brock 2007; Zhang et al. 2010). Because body size and age of females are correlated in painted turtles (Fig. 2.1b), body size is one reliable indicator that males may use to access female reproductive quality (Congdon et al. 2003; McGuire et al. 2011); however, assessment of female quality by male Painted turtles has not been documented.

Patterns of variation in multiple paternity

Over the four years of the genetic study, female age was positively associated with the probability of a clutch being sired by more than one male (multiple paternity). The increase in incidence of multiple paternity could result from an increase in the number of males attempting to mate with high quality (larger and older) females.

If the majority of adult males in the population are reproductive each year, the adult sex ratio will vary with the number of reproductive females. During 2003-2004 the numbers of reproductive females was low (Fig. 2.1a), the occurrence of multiple paternity was high in second clutch producing females and was positively associated with female age. When the number of reproductive females was high (2005-2006), multiple paternity was lower and not significantly associated with age than in 2003-2004. Both results suggest that when relatively more adult males are available per female, more mating attempts per female occur.

The difference in association of the incidence of multiple paternity with age in years with the high compared to low numbers of reproductive females may be due to a higher proportion of young females (generally individuals with low-reproductive qualities) being added to the number of reproductive females in a given year. A high number of young adults recruiting into the group of reproductive females in years with low total number of females may increase the number of males attempting to mate with high quality females.

Variation in repeat paternity

Reproductive frequency of females represents an important component of female quality (Gibbons et al. 1982), whereas reproductive frequency of females and repeat paternity of a female's clutches in combination are an important mechanism influencing male RS. Over 4 years sampled males mated with an average of a single female, therefore, increased male RS occurs as a function of female reproductive frequency when males sire offspring in subsequent clutches of females (i.e., repeat paternity). The majority of Painted turtle females apparently use stored sperm from the same male to fertilize eggs in second clutches within a year (McGuire et al. 2011) and in other populations (Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002). Additionally, repeat paternity occurs in ~ 40% of clutches produced among years in ESGR Painted turtles (Fig. 2.6) and also occurs in other species of turtles (Fitzsimmons 1998; Roques et al. 2006). Collectively, the results indicate that repeat paternity (through the use of the same male's stored sperm or by females or remating with the same male) contributes substantially to male RS.

The high incidence of repeat paternity among years coupled with variation in female reproductive frequencies and the importance of the number of clutches sired supports predictions that mate quality is more important than mate number to variation in male RS in the ESGR population (McGuire et al. 2011). Because both inter- and intra-annual reproductive frequency increase with female age and size (Congdon et al. 2003; McGuire et al. 2011) body size is a reliable indicator of female reproductive quality. However, the presence of age effects associated with multiple paternity without significant body-size effects suggests that additional cues in assessing female age must be present. If males can identify and successfully mate with high quality females, they will have substantially higher RS than males that mate randomly and the incidence of multiple paternity will increase among both older females and those that frequently produce two clutches in a year regardless of age.

The frequency of repeat paternity among years and the relatively low estimate of multiple paternity begs the question: why would some females sire offspring with the same males among years if males are plentiful? Iteroparity provides an opportunity for females to change mates between reproductive bouts, so we examined whether attributes of the males changed when paternity differed among-years. The "trading up" hypothesis predicts that remating will occur if a female subsequently encounters a higher-quality mate or mates (Uller and Olsson 2008). In Painted turtles, changes in paternity among clutches produced in different years exhibited no consistent pattern of increasing or decreasing body size or age with a "new" male compared to the male who sired progeny in a previous clutch.

Male reproductive success

The total number of offspring sired by sampled males varied among males within year (1-19 offspring) and among males over the four years (1-32 offspring; Table 2.1). Neither body size not age of the male was associated with variation in male reproductive success (Fig. 2.7). However, we did find evidence of assortative mating by age. The number of clutches a female produced coupled with repeat paternity was a substantial source of variation in male RS, whereas the number of females a male mated with was not. If there are males that can identify and mate with high quality females, they will on average leave more offspring compared to males that mate indiscriminately (Altmann 1997; Shine et al. 2003; Zhang et al. 2010).

Although male RS can be influenced by the presence of multiple paternity the incidence of occurrence in the present study averaged 14% over all years and was not a major determinant of male RS. However, in other species of turtles and in reptiles in general, the incidence of multiple paternity is much higher (Lee 2008; Uller and Olsson 2008) and therefore may be a larger source of variation in male RS than it is in painted turtles.

Painted turtles are often described as having a polygamous mating system. However, we found that over a four-year period, the vast majority of males successfully mated with a single female. The number of mates for males was rarely greater than 1, whereas females clutches were sired by have multiple males within and among years (mean number of mates 1.18 per year and 1.74 over 4 years). Our data suggest that at least in the ESGR painted turtle population, the mating system is more accurately described as polyandrous.

Why don't the majority of males mate with more than one female within or among-years? Several explanations are possible: 1) mating with multiple females may be costly particularly in the ESGR population where the adult sex ratio is 2.3 males per female, 2) if male courtship behaviors are prolonged or energetically expensive, or mate guarding is extensive, males may not be able to obtain additional successful matings opportunities, and 3) males may attempt to mate with several females, but female choice or post-copulatory sexual selection in the use of stored sperm may reduce the number of successful matings per male (Eberhard 1996). Post-copulatory sexual selection is widespread among taxa and has been documented in insects, birds, mammals, and reptiles (Birkhead and Pizzari 2002; Calsbeek and Bonneaud 2008). In the absence of behavioral data on mating tactics, genetic methods that allow paternity to be inferred precludes the ability to disentangle pre- and post-copulatory mechanisms leading to the outcome of paternity. Thus, the patterns of repeat paternity, change of paternity, and male RS in painted turtles could be due to pre- or post-copulatory mechanisms, and represents an area of research to be further explored.

Our findings have implications for other iteroparous organisms, particularly those species where females have the ability to store sperm and exhibit propensities for fertilizing subsequent clutches with sperm of the same male. If the number of females is limited, then the first priority should be securing any mate, but that tactic is not apparent in the ESGR population (i.e., almost all successful males mate with only one female). Our results highlight an example where male mate choice could evolve in the absence of male parental care, sex role reversal, or cooperative breeding (Trivers 1972; Clutton-Brock and Vincent 1991; Clutton-brock 1991; Hauber and Lacey 2005).

APPENDIX 2

Table 2.1. Summary of the number of offspring, clutches, and mates per year and over four years (includes residual paternity) for successful male Painted turtles (*Chrysemys picta*).

	Per Year			For Four Years		
	Mean	SD	Min-Max	Mean	SD	Min-Max
Number of Offspring	7.18	3.99	1-19	8.64	5.81	1-32
Number of Clutches	1.41	0.57	1-3	1.68	0.87	1-5
Number of Mates*	1.05	0.23	1-2	1.09	0.30	1-2

*Only one year (2005) had an average number of mates that was greater than 1 (1.15 mates/male).

Fig. 2.1. a) The number of reproductive Painted turtle (*Chrysemys picta marginata*) females per year (1983-2006) with yearly occurrence of multiple paternity (2003-2006) and b) relationship between body size and age of females.

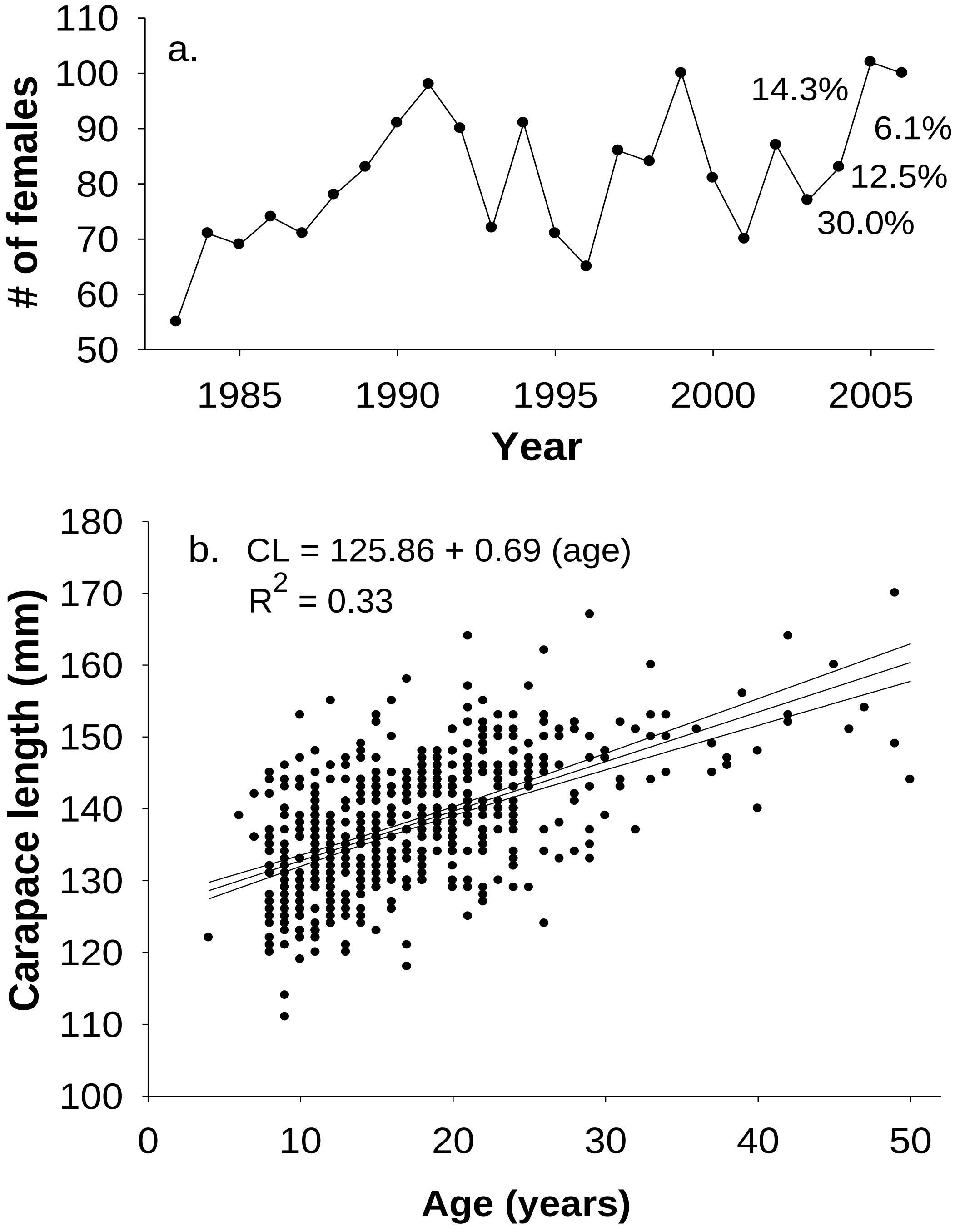


Fig. 2.2. a) Relationships between a) clutch size, and b) egg widths and body size of females, and c) frequency of second clutch production and age groups of females.

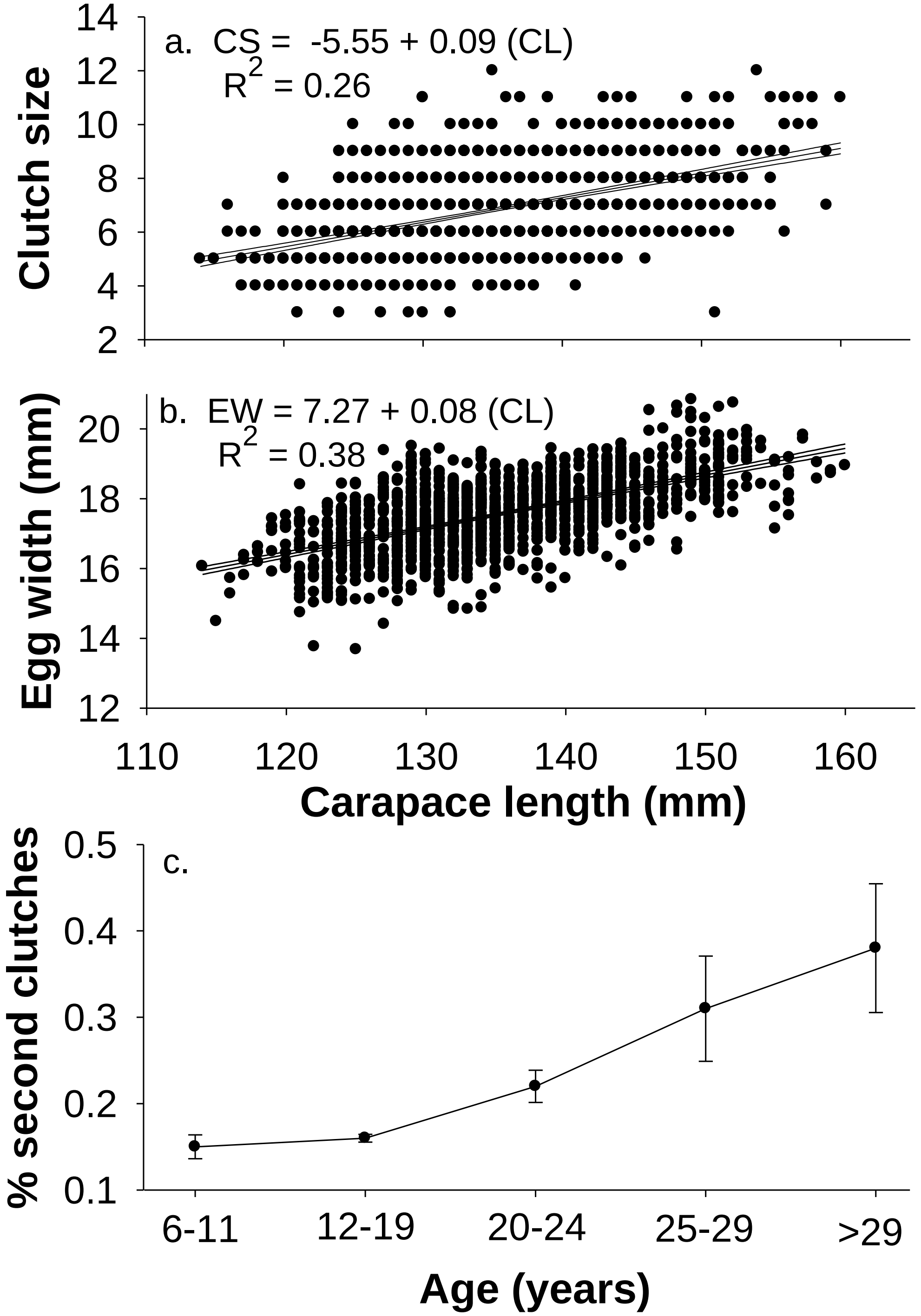


Fig. 2.3. a) Distribution of carapace lengths and b) ages of reproductive females from East Marsh captured in 1999 and reproductive females subsequently captured from 2000-2006 (14.6% and 51.8% of 1999 and 2000-2006 females respectively, were < 12 years old; the ages that females reach reproductive maturity).

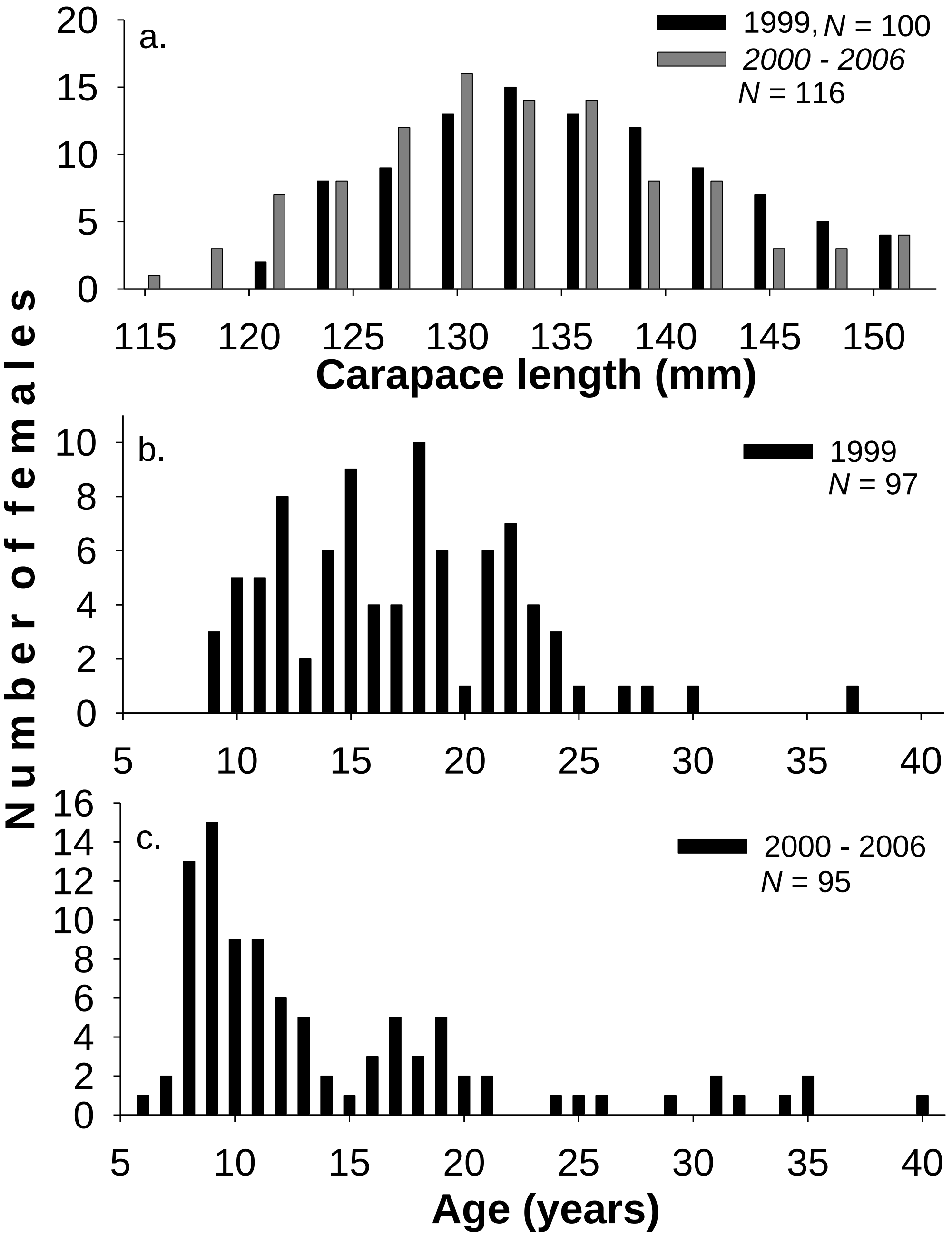


Fig. 2.4. a-f) Histograms of body sizes of females sampled over different periods of the life history and genetics studies.

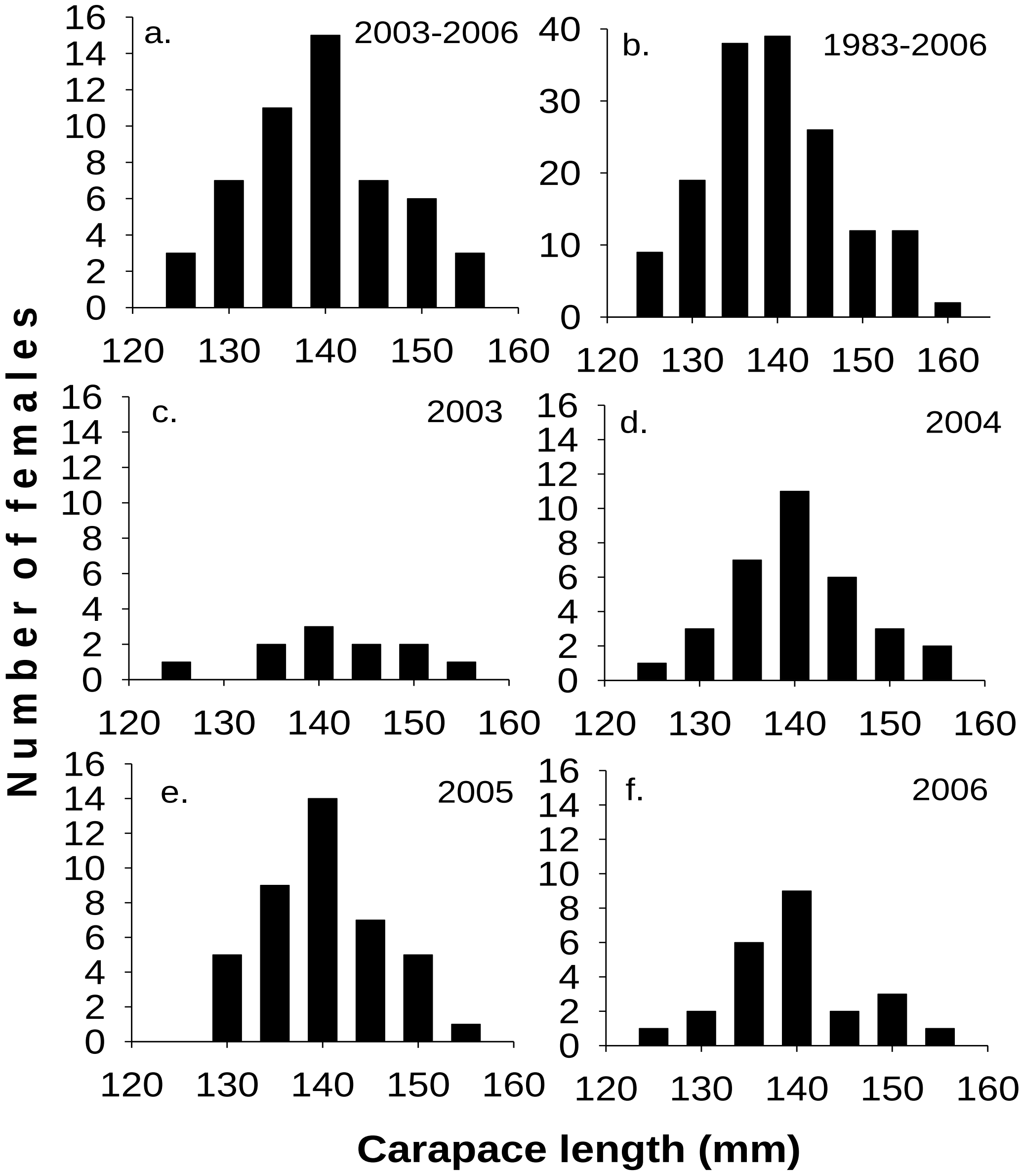


Fig. 2.5. a-f) Histograms of ages of females sampled over different periods of the life history and genetics studies.

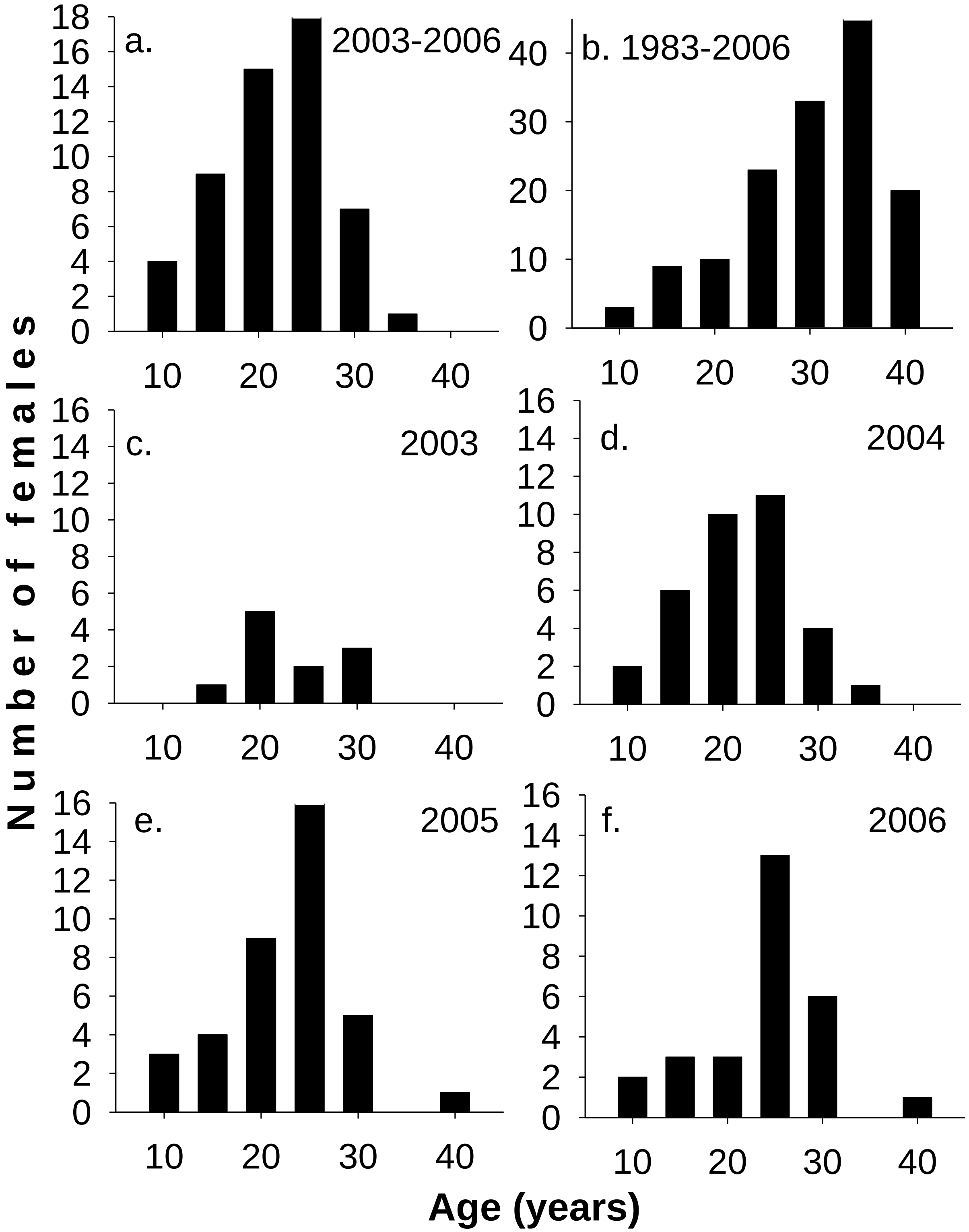


Fig. 2.6. Percent of nests with evidence of the same male siring offspring with the same female (repeat paternity) within- and among-years.

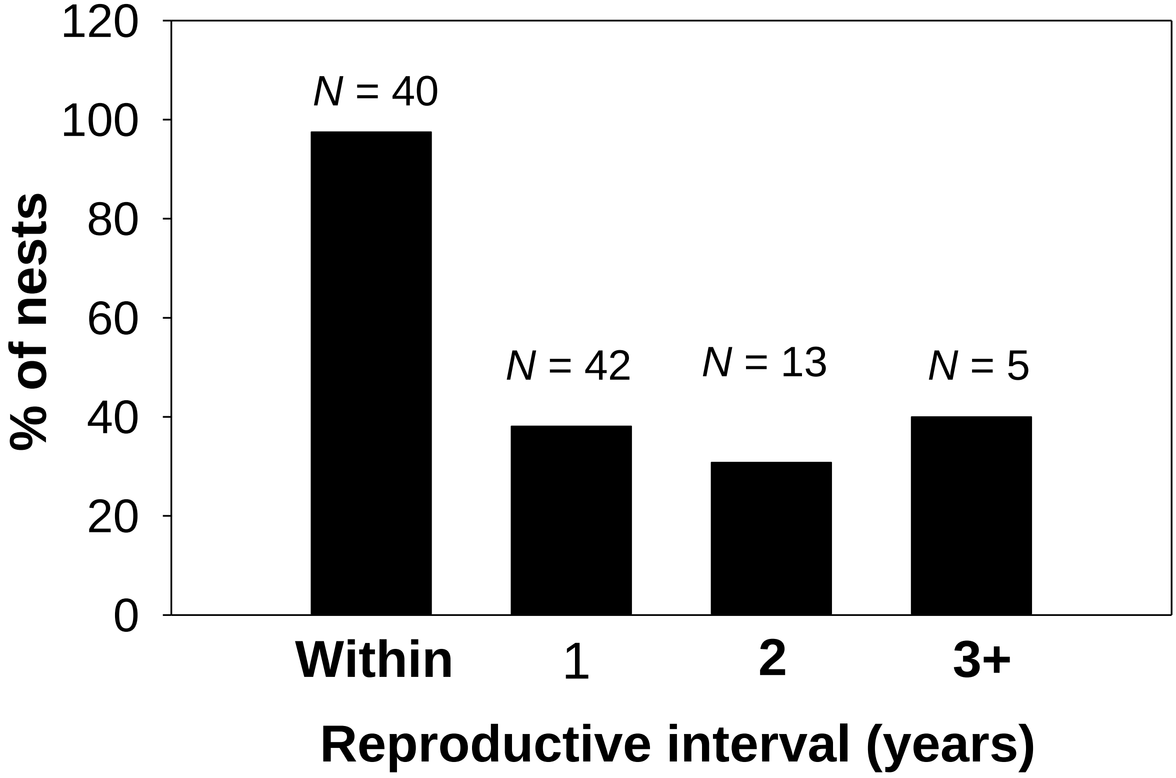
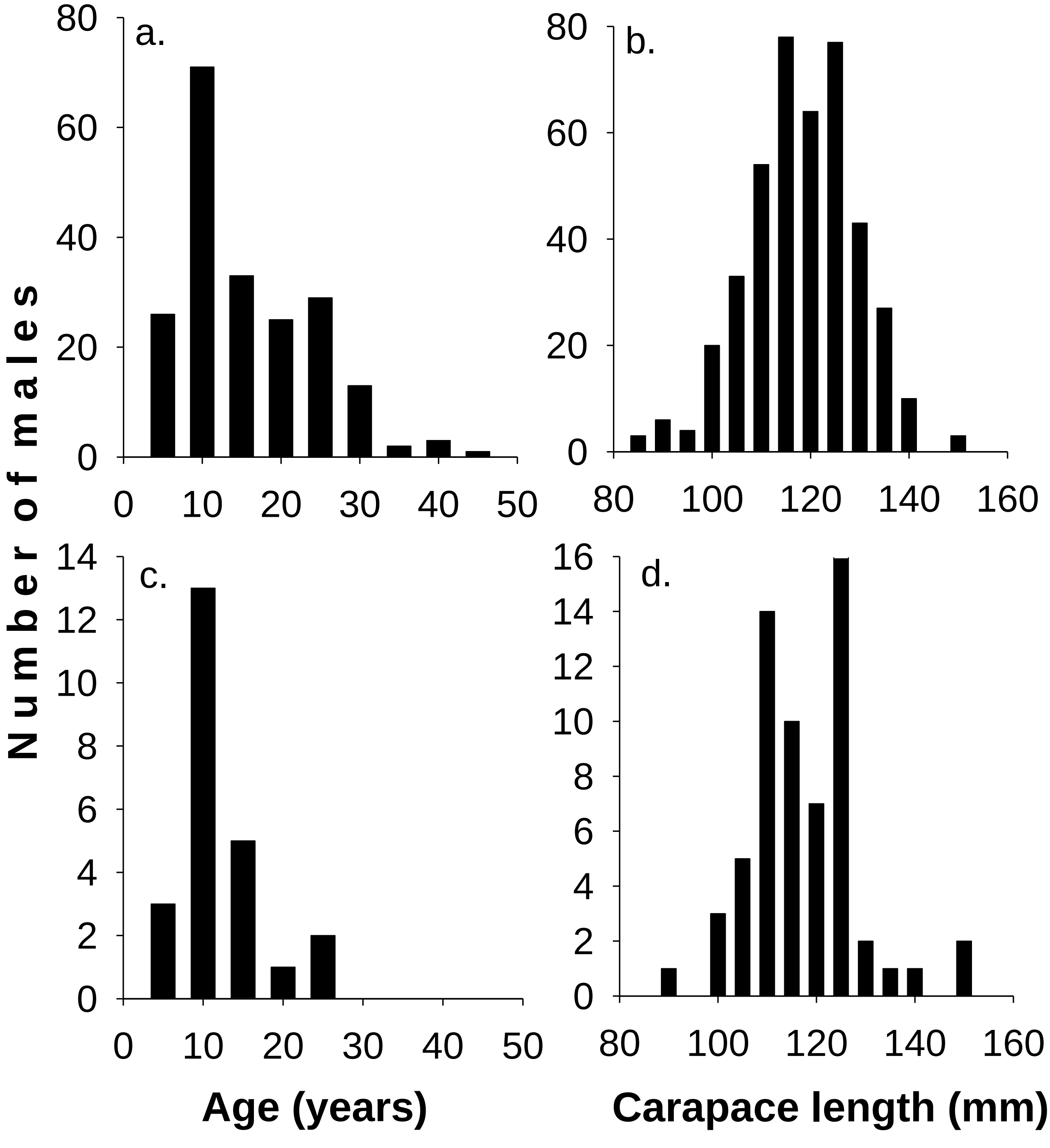


Fig. 2.7. Distribution by age and body size for all males sampled (a, b) and for males that mated successfully in the population (c, d) 2003-2006.



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CHAPTER 3: MATING SYSTEM, MALE REPRODUCTIVE SUCCESS, AND POPULATION CONNECTIVITY OF BLANDING'S TURTLES (EMYDOIDEA BLANDINGII)

Abstract

Identification of factors that facilitate or impede gene flow among populations requires an integrative approach, where multiple sources of information are simultaneously evaluated. Long-term data on population demography, reproductive ecology, and core areas were combined with genetic data to identifying mating pairs, and male reproductive success. We then examined spatial and genetic structuring among threatened Blanding's turtles (*Emydoidea blandingii*) inhabiting wetlands on and surrounding the Edwin S. George Reserve (ESGR) in southeastern Michigan. Analyses were based on data from nests of 49 females and inferred male paternity of 77 nests collected over 7 years (1999-2006). Nests located in terrestrial areas were usually nearer to residence than to non-residence wetlands; although nests were most often located in closer proximity to temporary wetlands. For resident ESGR females, 34.1% of clutches were sired by non-resident ESGR males, whereas 56% of clutches of non-resident females that nested on the ESGR were sired by ESGR resident males. Males and females had multiple mates (mean 1.56 and 2.02, respectively). Over four years, the occurrence of multiple paternity was 47.6% (min-max = 15.4%-55.6%). However, repeat paternity (same male siring offspring with the same female among years) was common (69.6% of inter-annual clutch pairs from the same female). Propensities of adults to mate with individuals from different wetlands and tendencies for hatchlings to disperse to wetlands other than the mother's residence wetland were two important but previously undocumented sources of gene flow among wetlands. Lack of significant differences in allele frequencies among individuals from different residence wetlands ($F_{st}=0.002$, $P > 0.05$) are concordant with observations of adult and juvenile movements and genetic data identifying mating pairs. Landscape-level conservation efforts should include large

areas of terrestrial habitat around residence wetlands to facilitate adult and juvenile movements and mating encounters that allow gene flow within and among populations..

Introduction

Quantification of the relative importance of factors that promote or limit inter-population gene flow can be improved by integrating data from multiple sources (Steen et al., in press). Direct observations of individual movements and habitat used to complete life functions (i.e., core habitat; Semlitsch and Bodie 2003) can be used to infer rates and direction of dispersal. However, in the absence of data on spatial variation in gene frequency or genetically determined parentage that identifies mating pairs, factors that affect gene flow cannot be directly quantified (Lowe and Allendorf 2010). Genetic data that allow identification of mating pairs and male reproductive success, when supported by information on movements of adults that affect mating encounters and of juveniles dispersing from natal areas can provide more definitive characterization of how aspects of species' reproductive ecology and habitat use throughout the year facilitate or impede gene flow. Such data are often lacking or are available only for limited periods of time. Thus researchers often lack the ability to generalize findings to other conditions (e.g., among years), locations, or species.

Rates of immigration and emigration among populations are influenced by constraints imposed by morphology, dispersal capabilities, risks, reproductive ecology, and the distribution of available of resources such as food and mates (Clobert et al. 2001). Such data are particularly important for semi-aquatic organisms such as turtles where core areas include permanent bodies of water, ephemeral wetlands, and terrestrial habitats surrounding wetlands (Semlitsch 1998;

Calhoun et al. 2003; Roe et al. 2009; Congdon et al. *2011*). Use of terrestrial areas and permanent and temporary wetlands by turtles can increase encounters and matings between individuals from different permanent wetlands. Therefore, use of landscapes between resident wetlands can facilitate gene flow (Calhoun et al. 2003; Congdon et al. 2011). For example, some species of turtles move among ephemeral wetlands and seasonally flooded areas to exploit resources and mate (Bodie and Semlitch 2000; Kinney 1999), and some females make pre-nesting movements up to 20 km along wetland corridors and overland (Obbard and Brooks 1980; Power 1989). Additionally, males and females move across upland areas to find mates (Morreale et al. 1984; Gibbons et al. 1990), visit ephemeral wetlands to exploit seasonally abundant resources (Bodie and Semlitsch 2000), and to locate overwintering sites (Buhlmann et al. 2009). Movements also occur when aquatic habitats become uninhabitable due to droughts (Cagle 1944; Gibbons et al. 1983; Morreale et al. 1984; Kennett and Georges 1990), which can result in episodic but high rates of gene flow (Scribner et al. 1995). Therefore core areas of turtles and other semi-aquatic vertebrates often extend well beyond a permanent wetland (Semlitsch and Bodie 2003; Congdon et al.2011).

Because observations of matings in turtles are rare, and do not necessarily indicate the copulating male sired offspring, genetic data are required to document the occurrence of multiple matings and successful matings between individuals from different wetlands. The frequency of such matings provide more informed predictions of levels of gene flow and population levels of genetic diversity. Genetic diversity can be influenced by the number and proportion of individuals that have more than one mate (Nunney 1993; Karl 2008) and variation in reproductive success (Pusey et al. 1997; Miller et al. 2009; Frankham 1995).

Extensive demographic, ecological, and life history data are rarely available for long-lived poikilothermic vertebrates (Avise 2001; Owens 2006), particularly those that are considered threatened or endangered throughout most of their range (Herman et al. 2002; Mockford et al. 2005; Mockford et al. 2007; Congdon et al. 2008). Blanding's turtle (*Emydoidea blandingii*) is a notable exception because it is one of the most studied species in North America. In many parts of their range, Blanding’s turtles exist in small and isolated populations that overlap with human development (Grgurovic and Sievert 2005). Individuals make extensive movements (Ross and Anderson 1990; Kinney 1999; Hamernick 2001; Calhoun et al. 2003) and home ranges and core areas are large (Hamernick 2001; Congdon et al. 2011). Females produce a maximum of one clutch per year, nest survivorship is low and variable (Congdon et al. 1983; Congdon et al. 2000), and hatchlings emerging from nests orient and disperse toward far dark horizons (Butler and Graham 1995; Pappas et al. 2000). Females do not reach sexual maturity until 14 to 20 years of age, and adult survivorship in some populations is > 0.97 (Congdon et al. 2001; Congdon et al. 2003). However, data on spatial genetic structuring and gene flow can have substantial influence on the way the extensive life history and ecology data are interpreted.

The life history and ecology of Blanding's turtle has been studied on the University of Michigan’s Edwin S. George Reserve (ESGR) near Hell, MI for 43 of the past 57 years (Owen Sexton, 1953-1957; Henry Wilbur, 1968-1972, Donald Tinkle 1975-1979, and Justin Congdon (1975-2007). During the last 33 years of continuous study, 2,081 individuals (excluding hatchlings) were marked, 5,800 recaptures were made, 1,050 x-radiographs of gravid females were taken, and the locations of 614 nests and fate of 433 nests of known females were recorded. Background data on nesting ecology and female reproductive success (Congdon et al. 1983, Congdon et al. 1983, 2000), movements and use of core areas (Kinney 1999; Congdon et al.2011), demography and life history (Congdon and van Loben Sels 1991; Congdon et al. 1993; Congdon and van Loben Sels 1993; Congdon et al. 2001) are well documented.

The general goal of our research was to identify factors that contributed to gene flow among Blanding's turtles that reside for decades in different wetlands (Congdon et al. 2011). We used long-term data to generate questions and predictions about how life-history traits and core-habitat use influence mating tactics and to test the predictions using genetic data. We quantified components of variation in male reproductive success in relation to: 1) number of mates, 2) mate quality (clutch size, clutch frequency, egg size), 3) occurrence of multiple paternity 4) the number of eggs a male sired per clutch, 5) repeat paternity (via stored sperm or remating), and 6) age and body size of both sexes of adults. We also quantified the proportion successful matings among individuals from different residence wetlands on and off of the ESGR.

Traits of Blanding's turtles and development of research questions

Female Blanding's turtles produce an average clutch of 9.9 eggs (min-max = 2-19, SE = 0.70), and inter-clutch intervals are greater than one year (i.e., some females do not reproduce each year; Congdon et al. 2001). Reproductive characteristics of female Blanding's turtles (i.e., clutch size, clutch frequency and egg size) vary as a function of body size and/or age (Congdon et al. 1983; 2000). Based on all captures on the ESGR, adult sex ratios among resident individuals is approximately 1:1. Females can store sperm for several years, and clutches of eggs are sometimes fertilized by more than one male (Refsnider 2009), traits that are similar to those of other emydid turtles (Painted turtles (*Chrysemys picta marginata*): Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002; McGuire et al. 2011, European pond turtles (*Emys obicularis*; Roques et al. 2006)

Because body sizes and ages of the majority of individuals on the ESGR are known, we can assess whether body size, or some phenotypic traits that vary as a function of age, are associated with differences in mating tactics and male reproductive success (Fig. 3.1, Question 1), and whether female reproductive qualities (clutch size and frequency) affect male reproductive success (Fig. 3.1, Question 2).

We predict that repeat paternity reflecting either female use of stored sperm or repeated matings will be frequent and thus will be an important component of variation in male reproductive success (Fig. 3.1, Questions 3 and 4), and may have a substantial influence on levels of gene flow among different wetlands. Variation in male reproductive success, multiple paternity, and female use of stored sperm can influence cohort and population levels of coancestry and inter-population variance in allele frequency (Scribner et al. 1993). Based on the ability of Blanding's turtles to store sperm and results from other studies of turtles demonstrating repeat paternity (Painted turtles: McGuire et al. 2011, Pearse et a al. 2001, Pearse et al 2002, Pearse and Avise 2001, European pond turtles: Roques et al 2006, Green turtle (*Chelonia mydas*), Fitzsimmons 1998),

Philopatry to residence wetlands and nests located relatively close to residence wetlands increases the probability of offspring recruiting to the mother's residence wetland, and thereby increases the probability of inbreeding (Fig. 3.1, Question 5). Adult Blanding's turtles of both sexes have occupied one of the two permanent wetlands on the ESGR for 40 or more years and very few adults changed residence (Congdon et al. 2011). Although copulations occur in residence and ephemeral wetlands (Kinney 1999), the proportion of successful matings that occur in each type of wetlands is unknown, as a result, genetic structuring between residence wetlands may or may not be present (Fig. 3.1, Question 6).

If males and females consistently use the same ephemeral wetlands, repeated encounters will increase the probability of repeat matings and repeat paternity between the same pair of individuals in different years (Fig. 3.1, Question 3). Further, use of the same ephemeral wetlands by resident and non-resident adults is a potential mechanism for gene flow through matings (Fig. 3.1, Question 7). Adult male and female Blanding’s turtles make extended terrestrial excursions to visit ephemeral wetlands where they can utilize seasonally abundant resources. Terrestrial movements also increase the probability of encountering adults from different residence wetlands (Baker and Gillingham 1983; Ross and Anderson 1990; Rowe and Moll 1991; Kinney 1999; Beaudry et al. 2009).

Nesting area selection and hatchling dispersal can contribute significantly to genetic exchanges among wetlands, reducing coancestry and probabilities of inbreeding (Fig. 3.1, Question 8). Some adult females on the ESGR make extensive terrestrial movements from residence wetlands to nesting areas (up to 2 km, Congdon et al., 2011) that result in some females leaving the ESGR to nest. As the distance between resident wetlands and nesting areas increases, the probability of hatchlings dispersing into the mother’s residence should decrease.

Methods

Field sampling of adults and determination of residence wetland.

Blanding's turtles on the ESGR were captured in aquatic traps, on land, and at drift fences during 44 of the 54 years from (1953-2007). At first capture, all juveniles and adults were given a unique identification mark by notching a sequence of marginal carapace scutes. At every capture, the date, individual identification, body size (carapace length, and weight), and reproductive condition (e.g., gravid or not gravid; females only) were recorded. To quantify traits associated with female quality, females with eggs were X-radiographed to determine clutch size and egg width (Gibbons and Greene 1979; Hinton et al. 1997). Hatchlings were given nest specific cohort marks and both hatchlings and yearlings were assigned "age 1". At first capture, juvenile ages were estimated from counts of growth rings, assuming one growth ring for each year of life (Gibbons 1976).

Age at maturity for females was determined from recapture histories and presence of eggs documented with X-radiographs. Ages at maturity for males were not known, but a minimum age at maturity was provisionally set at 21 years based on the asymptote of the relationship between age and body size in the ESGR population (Congdon and van Loben Sels 1993). Adults were assigned as ESGR residents based on extensive aquatic captures over more than four years in one of two permanent wetlands (East and West; 1.9 km apart) on the ESGR. Hereafter, 'residence' or 'residence wetland' will be used interchangeably. Females were assigned as non-residents based on extensive captures histories on land in ESGR nesting areas, but with minimal or no aquatic captures in ESGR wetlands over 33 years (Congdon et al.,2011).

Hatchling sample collection

Hatchlings of 49 marked females were obtained from 77 nests (611 hatchlings) from 1999-2006. Nests were protected *in situ* using wire cages to exclude predators except during 2005 and 2006 when eggs were transferred to a protected natural nesting area within four hours of nest completion and incubated in perforated plastic containers to exclude burrowing mammal predation. Samples from all years were combined for analyses.

Collection of genetic data

From 1997-2007, approximately 1cc of blood was taken from the caudal vein of the tail from all adults captured on the ESGR (males, *N* = 85 that were all residents of ESGR marshes; females, *N* = 163 including 32 ESGR residents and 131 non-residents) to use for genetic analysis. Based on annual catchability estimates of 85% (Congdon et al. 1983), nearly all resident males and females were captured every three years, and the probability that individuals were not captured over the duration of the genetic sampling (11 years) was very low. Blood samples from adults were taken during 1997-2006 and a small tissue sample (< 2 mm) was taken from the tail of hatchlings from protected nests from 1999-2006 for parentage analysis. All blood samples were stored at room temperature in a blood storage buffer (100 mM TrisHCL (pH 8.0), 100 mM EDTA, 10 mM NaCl and 0.5% SDS), and tissue samples were preserved and initially stored at room temperature in 95% EtOH and then refrigerated at (4°C). The DNA was extracted from blood and tissue samples using a Qiagen DNeasy kit (Qiagen Inc., Valencia, CA) and quantified using fluorimetry.

All individuals were genotyped at 8 microsatellite loci including BTGA5, BTCA11, BTCA9, (Libants et al. 2004), EB11, EB19, EB17, EB09 (Osentoski et al. 2002), and GmuD70 (King and Julian 2004). Polymerase chain reaction (PCR) was performed to amplify the DNA using published conditions and separated using gel electrophoresis on a 6% polyacrylamide gel. Products were visualized using an FMBIOII scanner (Hitachi Inc., Kanagawa**,** Japan). All gels were independently scored by two experienced laboratory personnel. To estimate error, and ensure consistency of allele assignment among samples, 10% of adults were randomly selected and re-genotyped. Ten percent of PCR products from hatchlings were also reanalyzed electrophoretically and any discrepancies resulted in re-amplification and and validation.

Statistical analysis

Genetic data were used for parentage assignment and evidence of spatial genetic structuring, which are both contingent on levels of genetic diversity and based on the assumption of Hardy-Weinberg equilibrium. All loci were tested for deviations from Hardy-Weinberg equilibrium, independence, presence of null alleles, measures of genetic diversity (average number of alleles and expected heterozygosity). Summary statistics and the multi-locus paternal exclusion probability were estimated using program CERVUS 3.0 (Kalinowski et al. 2007).

Paternity assignment

The use of multiple parentage programs helps to ensure a robust parentage assignment (Jones and Ardren 2003; Jones et al. 2007; Lee 2008; Wang and Santure 2009). Aspects of a species' biology, specific study objectives, and availability of other background information influences the most appropriate programs to use (Jones and Ardren 2003; Lee 2008). In this study, we observed females nesting so female parents were known for all offspring. Turtle offspring are produced as a group (clutch) and therefore the use of statistical methods implemented in programs that utilize clutch-level information (e.g. NEST; Jones et al. 2007) can enhance the accuracy of paternity assignment and reduce over-estimation of the number of contributing sires (Fiumera et al. 2002; Jones et al. 2007). In contrast to methods implemented in programs such as CERVUS (Kalinowski et al. 2007) that use a likelihood approach and considers each offspring as a random sample from the population, program NEST (Jones et al.

2007) uses a Bayesian framework that incorporates within-nest relatedness and prior information of paternity for individuals within the clutch to facilitate paternal assignment. We performed a two-tiered approach to assign a male to each offspring. We first used the program NEST to assign male(s) to a clutch, and subsequently assigned each offspring to a male using program CERVUS 3.0.1 (Kalinowski et al. 2007). Only in cases where the male(s) assigned by NEST also had a positive log odds (LOD) score estimated in CERVUS was a male assigned as a sire. When a sire was selected by one program but not confirmed with the second program, the sire was assigned as "undetermined". In cases where neither NEST nor CERVUS was able to assign a male(s), the nest was considered to be from an un-sampled (non-resident) male. Two or more males were determined to have sired offspring in a clutch (multiple paternity) based on assignment of two or more males or when a minimum of three paternal alleles were detected in the clutch. Repeat paternity (assignment of the same male to offspring in two or more clutches of a female in different years) was identified based on the same criteria. In cases where a sire could not be identified, the offspring in all sampled nests of the female parent were combined and re-analyzed using program NEST to assign offspring of a given female to one or multiple un-sampled males. If NEST assigned the offspring to the same un-sampled male(s), the clutch was also considered to be consistent with repeat paternity (sired by the same male).

Sources of variation in male reproductive success

We quantified sources of variation in male reproductive success using general linear mixed models (GLMM) using the lme4 and languageR packages in the statistical software R (Team 2007). Our dependent variable of male reproductive success was defined as the total number of offspring sired over the course of the study. Fixed effects associated with independent variables were quantified including male age and body size (for the years in which offspring were sired), total number of mates, total number of clutches sired, and whether the clutch was sired by multiple males (multiple paternity). Each fixed effect was tested independently in a mixed model that included female as a random effect to account for the fact that offspring from multiple nests were often available for the same female. Because some males mated with more than one female, and were therefore represented multiple times in the dataset, we also tested for factors associated with male RS using average values for male age, male body size, number of mates (per year and all years), number of clutches (per year and all years), and average clutch size.

Quantification of Gene Flow

We used parentage data to identify mating pairs and to quantify the occurrence of pairings of individuals from different resident wetlands (East and West). For each female, we compared female residence with that of her mate(s). For example, there were three categories of matings possible for an east residence female. An east residence female could produce offspring with an east-residence, a west-residence or non-resident male. More than one category may be present for a female in cases where she mated with more than one male (multiple paternity). The proportions of each category of matings were summarized by the residency of the female (e.g., the three potential categories for an east residence female should total 100%). Differences in the proportions of pairing were compared using adjusted G-tests.

Females were observed in the act of nesting during extensive searches of nesting areas. Nest locations were recorded by latitude and longitude for ESGR resident (east and west) and non-resident females using the program PASSaGE v2 (Rosenberg and Anderson 2011) and plotted using Google Earth. We used long-term capture-recapture data to identify recaptured hatchlings of known mothers and to examine whether the probability of hatchling recruitment into the mother's residence was associated with female nest location.

Evidence of Spatial Genetic Structure

We performed multiple analyses to characterize degree of spatial genetic structuring. First, based on *a priori* assignment of individuals to a residence category (east, west or non-resident), we used program FSTAT v4.0.11 (Goudet 1995) to quantify variance in allele frequency (F_{st}; Weir and Cockerham 1984) among individuals of each residence. Nominal alpha levels for the hypothesis $F_{st}=0$ were adjusted using a Bonferroni correction. Secondly, we estimated the number of genetic clusters using the program STRUCTURE v2.3.3 (Pritchard et al. 2000) based on all individuals (resident and non-resident males and females) sampled on the ESGR with no *a priori* information regarding residence. We performed 10 independent runs for each value of K = 1-4 using $3*10^6$ MCMC iterations after a $2*10^5$ step burn-in period. The most likely number of clusters was determined by identifying values of K that produced the highest log-likelihood value. If evidence for >1 genetic clusters was found, individuals were assigned to a cluster based on the highest posterior probability of cluster assignment.

Results

Variation in Female Reproduction

From 1976-2006, the total number of ESGR east, west resident and non-resident females that were captured on the ESGR were 34, 46, and 170 individuals, respectively. Although the proportion of the total nesting females over all years that were non-residents was larger than ESGR residents (G-test, $G = 66.27$, $P < 0.05$), annual captures of residents (mean = 12, min-max = 2-21), west residents (mean = 11, min-max = 5-19), and non-residents (mean = 11, min-max = 5-22; Fig. 3.2a) were similar. The numbers of resident and non-resident reproductive females captured each year were positively correlated (Fig. 3.2a; Pearson Rank Correlation; $\rho = 0.49$; $P = 0.005$).

We quantified two aspects of female quality (clutch size and egg size). Clutch size and mean egg size (width) of clutches estimated from x-radiographs of all reproductive females averaged 9.9 eggs (min – max = 2-19, SE = 0.70, N = 971) and 24.3 mm (min-max = 20.7-28.4; SD = 0.04; N = 963 clutches), respectively. Clutch size increased with female body size (CS = -8.25±0.095 carapace length; $r^2 = 0.26$, N = 280; (Congdon and van Loben Sels 1991), whereas egg size and reproductive frequency increased with increasing female age (Congdon et al. 2000). Mean carapace lengths of reproductive females each year (1976-2006; Fig. 3.2b,c) for east residents (mean = 211.1 mm, min – max = 186-217, SE = 2.18), west residents (mean = 203.1 mm, min-max = 196-210, SE = 2.21), and non-residents (mean = 196.6 mm, min max = 183-213, SE = 1.22). Resident and non-resident females that were part of the genetic study did not differ in CL, age, clutch size, or egg widths (GLM, CL, t = 0.24, df = 48, $P = 0.81$; age, t =1.45, df = 38, P=0.16; clutch size, t = -1.24, df = 33, $P = 0.22$; egg width, t = 0.42, df = 36, $P = 0.68$).

Genetic Diversity

All loci were in Hardy-Weinberg Equilibrium (HWE; $P > 0.10$). Loci were independent (no evidence for gametic disequilibrium, $P > 0.10$) and polymorphic (average of 7.4 alleles per locus, min - max = 4 - 15 alleles). Multi-locus expected heterozygosity was estimated to be 0.69 (min - max across loci 0.54 - 0.85). The multi-locus paternal non-exclusion probability with one parent known was estimated to be 0.004. There was no evidence of null alleles at each locus ($P > 0.10$). The multi-locus error rate was empirically estimated to be less than 1% across all loci.

Mating System and Reproductive Success

Females mated with multiple males within and among years and the within year average number of sires per clutch was 1.5 ($SD = 0.67$, min-max = 1-3). Over the 8 years of the genetic study, the average number of mates for a female was 2.02 males ($SD = 1.05$, min-max = 1-5) per female. However, because some females were sampled more frequently than others, we also adjusted the total number of males per female by the number of clutches sampled, after which the number of sires per female averaged 1.36 ($SD = 0.70$, min-max = 1-3).

Occurrence of multiple paternity was 41.6% over all clutches but variable among years ($N = 77$ nests; $N = 49$ females (27% of reproductive females); min-max = 15.4% - 55.6%; Fig. 3.2a). The incidence of multiple paternity was positively and significantly associated with female age (Question 1: GLMM; $Z = 2.037$; $P = 0.042$), body size (carapace length; GLMM; $Z = 2.19$; $P = 0.03$), and clutch size (GLMM; $Z = 1.998$; $P = 0.046$). Incidence of multiple paternity was similar for clutches of resident and non-resident ESGR females (GLM; $Z = 1.238$; $P = 0.216$). The total number of reproductive females each year was negatively correlated with the yearly incidence of multiple paternity (Pearson's Rank Correlation; $\rho = -0.857$, $P = 0.006$).

Males that successfully mated with sampled females averaged 1.56 mates (SD = 0.91, N = 32, min-max = 1-4) over the 8 years of the study. Due to the occurrence of repeat paternity, the average number of clutches sired over the 8 years was higher (mean = 1.97, SD = 1.53, N = 32, min-max = 1-7). The average number of offspring sired over 8 years was 11.22 (SD = 8.62, N = 32, min-max = 1-40).

Among males that successfully mated with sampled females that nested on the ESGR, male reproductive success was significantly associated with the number of mates (GLMM; t = 6.042; $P < 0.001$) and the number of clutches (GLMM; t = 13.091; $P < 0.001$). Male reproductive success was not significantly associated with male age (GLMM; $t = 0.150$; $P = 0.88$), size (carapace length; GLMM; $t = 0.618$; $P = 0.54$), offspring sired per clutch (GLMM; t = 0.091; $P = 0.928$) or multiple paternity (GLMM; $t = -1.846$; $P = 0.08$). Because males mated with more than one female, and were therefore represented multiple times in the dataset, we also tested for factors associated with male RS using average values for all fixed effects in the model. There were no differences between the use of the averaged and non-averaged fixed effects (male age, size, number of mates per year and all years, number of clutches, and average clutch sizes). The average number of mates per year, clutches per mate over all years, and offspring per mate over all years were significant predictors of male reproductive success (GLMM; average mates per year, t = 4.40, $P < 0.001$; average clutches per mate, t = 4.57, $P < 0.001$, average offspring per mate t = 4.05, $P < 0.001$). Male reproductive success was not influenced by average female body size, average female age, average number of offspring sired per clutch, or multiple paternity (Questions 2 and 4: GLMM; average age, t =0.44, $P = 0.67$; average CL, t =1.02, $P = 0.32$; average offspring per clutch, t =1.16, $P = 0.26$, average multiple paternity; t = 0.04, $P = 0.97$).

The size distributions of successful males and all genetically sampled males were similar (Fig. 3.4; X^2 = 6.44, df = 18, P = 0.994). However, age distributions of successful and all sampled males differed significantly (Fig. 3.4; X^2 = 11.42. df = 5, P = 0.044). The youngest successful male was 22 years of age.

Nests of 28 (57%) of 49 sampled females from 1999-2006 were sampled at least twice. There was evidence of repeat paternity (Question 3) or different males in 7 and 8 clutches, of 28 females, respectively, whereas evidence from clutches of 13 females was inconclusive. Of 23 pair-wise comparisons of each female's nests among years, 69.6% showed evidence of repeat paternity. Clutches of females reproducing in consecutive years had the highest incidence of repeat paternity. Evidence of repeat paternity occurred over clutch intervals extending over the four years of study (7 years; Fig. 3.5).

Gene flow estimated from paternity data

Resident females (N = 44) mated most often with males from the same residence wetland (G- test = 9.06; $P < 0.05$; Table 3.1). The proportion of clutches of non-resident females (N = 46) sired by resident and non-resident males did not differ significantly (G-test = 1.57, $P > 0.05$). However, non-resident females (N = 46) mated more often with west resident males than with east resident males (G-test, G= 18.69, $P < 0.05$). No other comparisons were significant (Table 3.1). Resident and non-resident females had similar probabilities of having at least one offspring in a clutch sired by a non-resident male (Questions 5 and 6: GLM; Z = -0.355; df = 30; P = 0.723). Regardless of residency, the probability of having at least one offspring in a clutch sired by a non-resident male was positively associated with female age (Question 7: GLM; $Z = 2.76$; df = 46; $P = 0.006$), but not body size (GLM; Z = 1.45; df = 50; p= 0.146).

The location of nests of east-resident females were generally nearer to their residence than to the west-residence wetlands (Question 5), indicating wetlands may be spatially genetically structured if offspring recruit primarily to the mother's residence. However, non-resident females nested throughout the reserve (Fig. 3.3).

Data from mother-offspring pairs (N=32) identified from the long-term mark-recapture data indicated that dispersal of offspring from the mother's resident wetland may contribute to gene flow among wetlands (Question 4). Half of the offspring (16) were from resident females (8 east, 8 west) and half (16) were from non-resident females; and 31 (96.9%) of the 32 offspring, were from nests located in the central portion of the ESGR.

Population and Genetic Structuring

Genetic analysis of all individuals sampled on the ESGR (west resident, east resident and non-residents) revealed that there is only a single genetic cluster (Question 9: STRUCTURE, K = 1, log-likelihood = -5132.0). Estimates of variance in allele frequency among females sampled from resident and non-resident wetlands were not significantly different from zero (F_{st}=0.002, $P > 0.05$).

Discussion

Females and reproductive characteristics.

Reproductive females from residence wetlands located on and off the ESGR were captured frequently while nesting on the ESGR. Although the average number of east-resident, west-resident, and non-resident females captured each year from 1976-2006 were similar (Fig. 3.1a), the total number of all resident females (*N* = 80) was about one half of the number of non-residents (*N* = 170) that could have originated from up to six permanent wetlands within 1.5 km (1.77 km^2 area) of the ESGR (Congdon et al., 2011).

Mating system and Reproductive Success

Mate number can influence the effective population size and population levels of genetic diversity (Sugg and Chesser 1994). Additionally, when clutches of eggs have multiple sires (multiple paternity), offspring within the clutch are a mixture of full- and half-siblings. Because offspring from multiple-paternity nests are genetically more diverse (i.e., lower coancestry; Scribner and Chesser 2001), the offspring from clutches sired by multiple males represent potentially important sources of genetic variation. Thus, it is important to understand the circumstances that contribute to the frequency of multiple matings by females. Both sexes of Blanding's turtles on the ESGR mate with more than one individual and mate numbers of males and females were similar (mean 1.56 and 2.02, respectively). The occurrence of multiple paternity in clutches of eggs on the ESGR (41. 6%) was lower than that found in population in Minnesota (56%; Refsnider 2009). Both estimates of multiple paternity in Blanding's turtles were higher than have been observed in other freshwater species including Painted turtles (Pearse et al. 2002, McGuire et al 2011), and the European pond turtle (Roques et al. 2006), and are more similar to species with substantially higher clutch sizes such as Snapping turtles

(*Chelydra serpentina,* Galbraith et al. 1993) and sea turtles (Green turtles, *Chelonia mydas,* Fitzsimmons 1998).

Multiple Paternity .-- The extensive movements of Blanding's turtles may also contribute to a high incidence of multiple paternity (Ross and Anderson 1990; Hamernick 2001; Grourovic and Sievert 2005). Two non-mutually exclusive movement-based mechanisms can affect multiple paternity. First, encounters with different individuals are more likely to occur if adults visit ephemeral wetlands widely distributed throughout the landscape compared to adults that remain in a single residence wetland. Second, if the dispersal of individuals to ephemeral wetlands decreases how often suitable mates are encountered, females may become less selective, and mate indiscriminately with males when they are encountered (Kokko and Mappes 2005). In contrast, an increase in the total number of males encountered may increase levels of female choosiness and can decrease multiple matings by females (Kokko and Mappes 2005; Sztatecsny et al. 2006; Rankin and Kokko 2007).

Overall, clutch size was positively associated with the frequency of multiple paternity. However, it is unlikely that the relatively high occurrence of multiple paternity in Blanding's turtles could be attributed solely to detection bias caused by clutch size variation among species. Clutch size in Blanding's turtles is more similar to those in other Emydid turtles (average 9.9 eggs) and smaller than those of snapping turtles (average on the ESGR of 20.8 eggs) or green sea turtles (>100 eggs; Fitzsimmons 1998).

The incidence of multiple paternity was variable among years (15.4% - 55.6%; Fig. 3.2) and was negatively correlated with the number of reproductive Blanding's turtle females. A similar relationship also occurred in Painted turtles, but only among females with the highest reproductive qualities (McGuire et al.2011. A relationship between multiple paternity and estimated breeding population sizes or density was found among species of sea turtles (Ireland et al. 2003; Jensen et al. 2006). Based on the data provided in Jensen et al. (Table 3.1, 2006), the mean occurrence of multiple paternity for species characterized by synchronized nesting was higher than in species characterized by solitary nesting (75.0%; min-max = 58-92%) compared to 30.6% (min-max = 9-50%), respectively; a difference of 44%. The authors caution that multiple paternity also varies from 9-62% among populations of a single species. Our results document among-year variation in multiple paternity within a Blanding's turtle population, (min-max = 15.4- 55.6%; a difference of 40.2%). Among year variation in multiple paternity in Blanding's turtles demonstrates that when levels of multiple paternity are estimated from different populations sampled in different years, interpretations of differences may be confounded by multiple sources of variation.

The age and body size of female Blanding's turtles on the ESGR were positive predictors of incidence of multiple paternity (Question 1). Reproductive frequency and parental investment (egg size) primarily increase as a function of female age, whereas clutch size (egg number) primarily increases with body size (Congdon et al. 2001). If males are able to identify larger or older females, they should increase efforts to mate with them; a result that could lead to a higher occurrence of multiple paternity in high quality females. Additionally, if larger or older females move more frequently or longer distances, they may have a higher probability of encountering and mating with more males.

Male reproductive success.--Females appear to mate indiscriminately with respect to male size and age. We found no evidence of male traits associated with male reproductive success. Male attributes such as body size and age were not significant predictors of male reproductive success

(Fig. 3.4). Additionally, the youngest male to sire offspring was 22 years old, an age that is consistent with estimates of age of maturity based on growth trajectories (Congdon et al. 2001).

Variation in reproductive success can accentuate the loss of genetic variation in the population because some individuals contribute disproportionally to the population (and in some situations some males may not contribute at all (Frankham 1995; Nunney 1996). Based on reproductive data from females that nested on the ESGR, we documented sources of variation in male reproductive success. The total number of offspring sired was variable among males (min-max = 1-40) and was significantly associated with the number of mates and the number of clutches sired, but not with multiple paternity (Question 2). Because the number of mates was a positive predictor of male reproductive success, pairings of males with multiple females can increase levels of coancestry among offspring from different clutches (Chesser 1991).

Male reproductive success in many species has been shown to be primarily determined by the number of mates (Bateman 1948; Clutton-Brock and Vincent 1991; Hauber and Lacey 2005). However, when substantial variation occurs in female reproductive qualities, female quality can also contribute significantly to male reproductive success (Darwin 1871; Arnold and Duvall 1994; Owens and Thompson 1994; McGuire et al. 2011). Our data on Blanding's turtles indicates that male reproductive success was influenced by the number of female mates and the number of clutches sired through remating or use of stored sperm by females (Question 4).

The number of years that viable sperm can be used by Blanding's turtle females has not been documented; however in ESGR Blanding's turtles, repeat paternity was observed throughout the duration of the study (maximum of 7 years between clutches). Additionally, the number of offspring sired per clutch did not necessarily decline among year (e.g., 9/9 (100%) and 10/10 (100%) despite a six-year interval; Question 3). Female turtles use stored sperm to fertilize sequential clutches within, and potentially among years (Ewing 1943; Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002; Roques et al. 2006; McGuire et al. 2011). However, use of stored sperm among years is associated with decreased fertility (Cuellar 1966; Jun-Yi 1982), that may result from sperm depletion or declining sperm viability (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998). In captivity, Eastern Box turtles (*Terrapene carolina carolina*) females produce viable offspring for up to 4 years after removal from males (Ewing, 1943). Our observations of repeat paternity extending beyond 5 years without declines in the proportion of offspring sired (e.g. 100% of offspring sired in clutches 6 years apart), suggests that females may have remated with the same males in different years. Co-occupancy of habitats (residence wetlands, overwintering sites, or ephemeral wetlands) could bring the same individuals together repeatedly among-years. Regardless of mechanism, repeat paternity will increase levels of relatedness (coancestry, Scribner et al. 1993) among hatchlings cohorts produced in different years and potentially increase levels of spatial genetic structuring (Scribner and Chesser 2001).

Gene Flow

Although resident females (east and west) mated predominantly with individuals from their same residence wetland, some successful matings occurred between non-residents and residents of the ESGR. Approximately one-third of east-resident and west-resident females mated with a non-resident male (Table 3.1; Question 6). Non-resident females nesting on the ESGR mated predominantly with other non-resident males and males from the west-residence. Fewer successful matings occurred between non-resident females and east-resident males (Table 3.1). Matings were observed in permanent and ephemeral wetlands on the ESGR during most of the activity season (Kinney 1999), and the genetics data from this study document matings between individuals from different residences on the ESGR and between resident and non-resident individuals. Combined, the behavioral and genetics data support that successful matings occur in ephemeral wetlands where encounters with individuals from different residences can occur (Question 7). Males and females may utilize ephemeral areas to exploit seasonally abundant resources (such as food) as well as to find mates (Kinney 1999; Pappas et al. 2000 Newton and Herman 2009; Congdon et al. 2011). Our data are consistent with the hypothesis that in addition to resources, males also move long distances to encounter mates. Regardless of wetland of residence, older females were more likely to have offspring sired by a non-resident male. Older individuals may utilize larger core areas or they may have gained experience that allows them to more efficiently exploit resources in ephemeral wetlands, and that in turn may expose them to a greater number of potential mates. .

Females from east and west residence wetlands primarily nested in the eastern and western areas of the ESGR, respectively (Fig. 3.3; Question 8). Therefore, the spatial distribution of nests indicates a potential for spatial genetic structuring between east- and west-resident individuals. Although the closest permanent wetland to nests may be the residence wetland of their mother, ephemeral wetlands (that might lead to dispersal of hatchlings away from residence wetlands) were much closer to many nests. For 32 ESGR resident mother-offspring pairs, 16 (50%) recaptured hatchlings came from non-resident females that that nested on the ESGR Also, because 31 of the 32 parent-offspring pairs were from nests located in the interior of the ESGR, nest-placement influenced the probability of juvenile recruitment into their mother’s residence wetland. Offspring of resident females that exit the ESGR to nest would also be expected to have a low probability of dispersing into ESGR residence wetland. Patterns of hatchling dispersal to residence wetlands other than the parents residences can homogenize allele frequencies among residence wetlands (Question 8).

Population and genetic structuring

Results from the genetic analysis of population structuring (STRUCTURE; k=1) demonstrated that individuals captured on the ESGR (residents and non-residents) represent a single population and thus the population of Blanding's turtles extends beyond the boundaries of the ESGR (Question 9). The lack of spatial genetic structuring is consistent with the occurrence and frequency of matings between males and females from different residences and with the probability that hatchling emerging from nests will disperse to wetlands other than their mother's residence.

Conservation implications

Adult Blanding's turtles exhibit a propensity for long-distance terrestrial movements and have the capacity to remain in terrestrial habitats over long periods (Power 1989; Ross and Anderson 1990; Rowe and Moll 1991; Kinney 1999). Extensive movements made by adults, high frequency of matings between resident and non-resident individuals, and high probability of offspring to disperse from nests to permanent wetlands other than the parents residences constitute two important but previously undocumented mechanisms that contribute to gene flow among wetlands. Therefore, population boundaries extend well beyond permanent wetlands and adjacent terrestrial areas suggested from the data collected during the long-term life history and nesting ecology study on the ESGR.

Clutches of older females had a higher probability of being sired by multiple males and by males that were non-residents. Therefore older females represent an important subset of the population, not only because of their comparatively higher reproductive frequency and offspring quality (Congdon et al. 2001), but also as an important means of distributing genes from multiple males to offspring thereby reducing cohort levels of coancestry (Scribner and Chesser 2001). In addition to the demographic value of older females to population dynamics and persistence (Congdon et al. 1993), the loss of older females would also lead to increasing population levels of coancestry and inbreeding that also influence population persistence. If there are age-based differences in movements, greater exposure of older females to human-induced disturbance that increase risk of mortality (such as roads) will substantially increase mortality thereby decreasing the mean age in the population (Gibbs and Shriver 2002) and concomitantly decreasing rates of gene flow among wetlands and increasing relatedness among offspring within and among year cohorts.

Blanding's turtles are characterized by polygamous mating system, with both males and females mating with multiple mates within and among years. The high percentage of multiple paternity is consistent with previous work in other turtle species (and other taxa) demonstrating that as male density declines, female choosiness may decrease, resulting in higher incidence of multiple matings. Females apparently make temporary visits to ephemeral wetlands to exploit seasonally abundant resources, whereas mate acquisition may be a more important factor than resources for similar movements by males. Repeat paternity resulting from female use of stored sperm or remating can accentuate loss of genetic diversity if multiple mates, and mates from other residencies, are no longer encountered. Given the species' propensity for frequent and extensive terrestrial movements, the integrity of the aquatic and terrestrial habitats are required to allow long-distance movements of all ages among wetlands that are crucial to the persistence of populations.

The results from this metapopulation case study highlight the importance of utilizing multiple sources of data to ascertain mechanisms of inter-population gene flow. Interpretations based on information on population size and core area gathered using a single method could have resulted in inaccurate conclusions, that could in turn misdirect management decisions. For example, data on fidelity to residence wetlands and the distribution of nests suggested that there was population structuring within the ESGR. However the inclusion of data on paternity, male reproductive success, mating pairs, and the degree of spatial genetic structuring led to a better characterization of the population. Similarly, analyses of genetic data indentified mating pairs and the spatial context in which encounters between males and females from different residence wetlands would not have been possible without long-term life history and demography data.

The combination of multiple sources of data identified several factors that influence gene flow. The higher reproductive output of older females coupled with increased multiple paternity make them demographically and genetically valuable compared to young females. The placement of nests of all females in areas that facilitate hatchling dispersal into wetlands other than residences of mothers, and the propensity of adult males and females to utilize large areas of terrestrial habitats surrounding ephemeral wetlands and mate with individuals from outside their residencies were all important sources of gene flow. In combination this study and that of (Congdon et al. 2011) demonstrate the importance of geographically expansive core areas of Blanding's turtles to successful conservation and management efforts. Protection of the core areas of Blanding’s turtles will also likely aid in the conservation of the majority of other semi-aquatic species.

APPENDIX 3

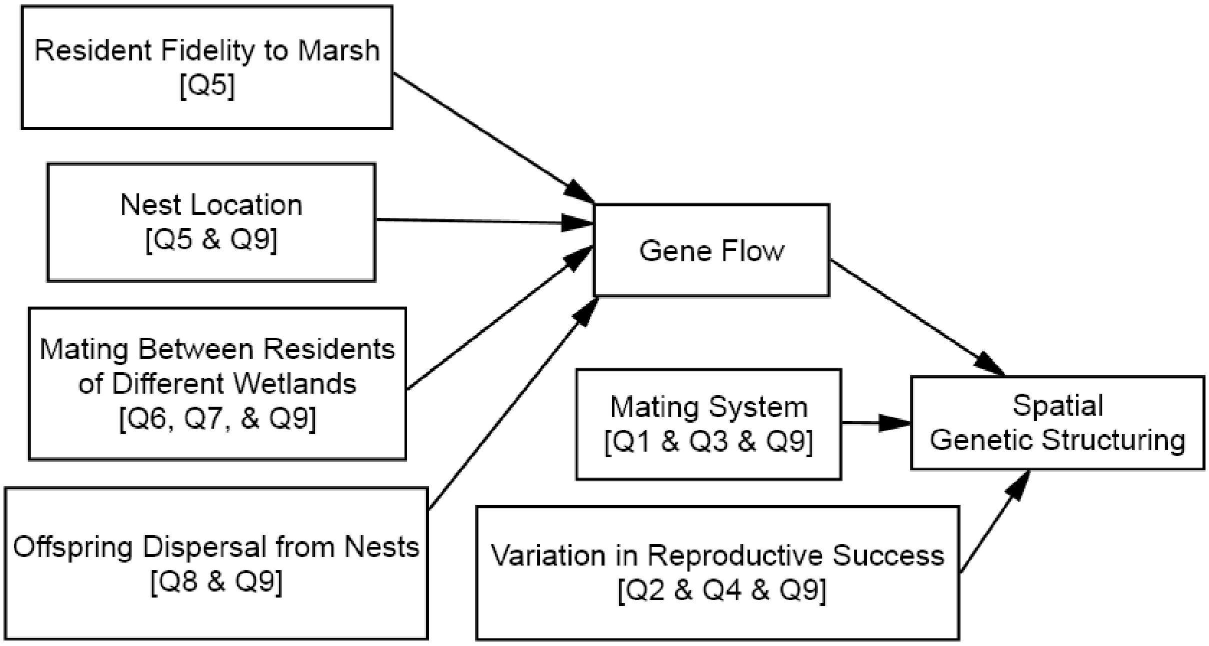
Table 3.1. Percents of matings (*N* = number of pairings) described during all years (2003-2006) between males and females with respect to residence wetland (East, West, and non-resident).

Residence wetland of females		Residence wetland of males		
		East	West	Non-resident
	East (33)	57.6	9.1	33.3
	West (11)	0.0	63.6	36.4
	Non-resident (46)	8.7	47.8	43.5

Table 3.2. Percentages of among-year clutches produced by females where repeat paternity was observed (*N* = number of comparisons) as a function of male and female marsh of residence (e.g. repeat paternity occurring between east-resident females east-resident males).

		Male residence wetland		
Female residence wetland		East	West	Non-resident
	East	64.3 (14)	25.0 (4)	50.0 (6)
	West	NA	33.3 (3)	100.0 (1)
	Non-resident	100.0 (1)	44.4 (9)	0.0 (3)

Fig. 3.1. Description of an integrative approach combining multiple sources of long-term data used to answer research questions [Q] pertaining to the relative importance of nesting ecology and movements, characteristics of mated pairs, and male reproductive success, to gene flow and spatial genetic structuring.



[Question 1] Are offspring from clutches of older or larger females sired more often by multiple males (multiple paternity) than clutches of offspring of younger or smaller females?

[Question 2] Is female reproductive quality (number of clutches, number of offspring per clutch) a substantial source of variation in male reproductive success (total number of offspring sired).

[Question 3] Are clutches of a female fertilized by the same male(s) in different years (repeat paternity)?

[Question 4] Does repeat paternity contribute significantly to male reproductive success?

[Question 5] Does long-term fidelity to residence wetlands and nest placement by females contribute to spatial genetic structuring?

[Question 6] Does fidelity to a residence wetland promote mating with males from the same marsh or influence repeat paternity?

[Question 7] To what extent does terrestrial movements of males and females facilitate matings among individuals from different resident marshes?

[Question 8] Does nest location influence whether offspring recruit into the resident marsh of the mother or disperse to another marsh?

[Question 9] Does repeat paternity and matings with individuals from different resident marshes, coupled with variation in nest placement by females among years, an important source of movements of male genes among marshes?

Fig. 3.2. The (a.) numbers of resident, non-resident and total reproductive females captured each year (1983-2006) on the E. S. George Reserve and ranks of the percent of nests with multiple paternity from 1999–2006 (% multiple paternity for each rank was 1 = 15.4, 2 = 36.4, 3=42.9. 4=44.4. 5 = 50.0, 6 = 53.8, 7 = 55.6; average carapace length (± 1SE) by year of (b.) resident and (c.) non-resident reproductive females.

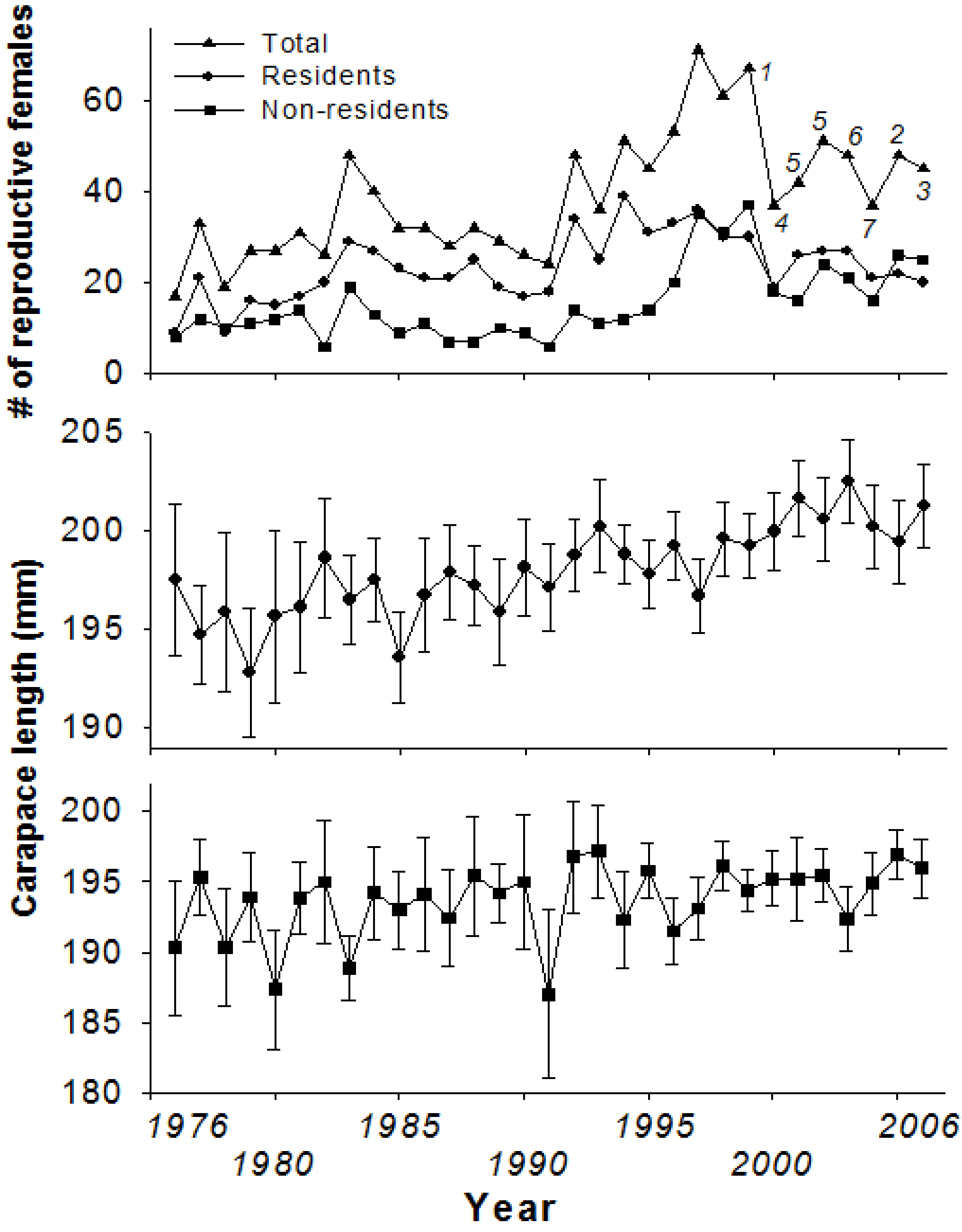


Fig. 3.3. Distribution of Blanding's turtle (*Emydoidea blandingii*) nests on the 650 ha Edwin S. George Reserve (boundary in dashed lines) from 1999-2006, residence wetland of females with the number of nests per location are indicated within symbols (circles = east residents, squares = west residents, triangles = non-residents of the E. S. George Reserve.

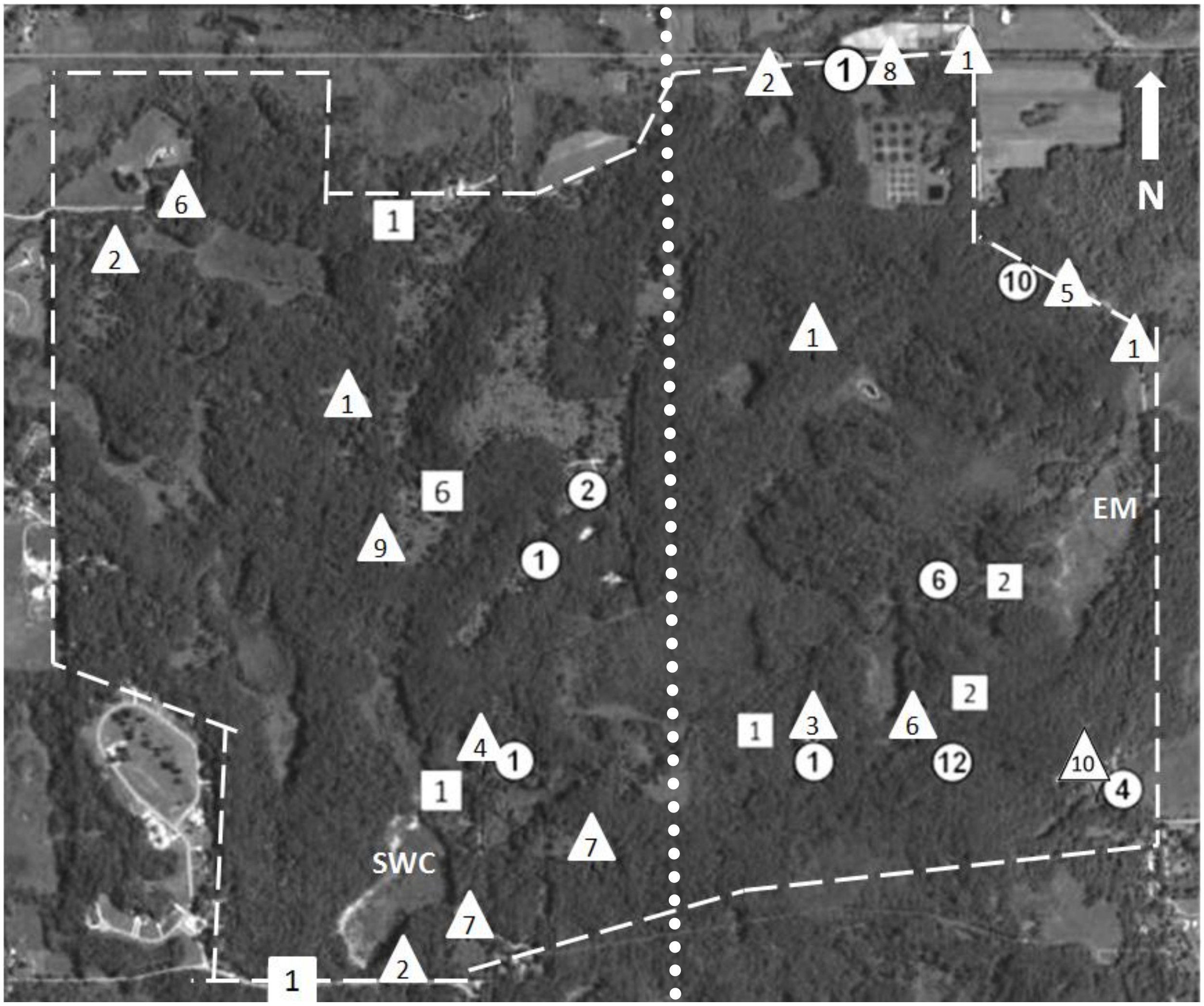


Fig. 3.4. Frequency histograms of (a) ages and (b) body sizes of males that successfully mated with females that nested on the E. S. George Reserve (ESGR) and the (c) ages and (d) body sizes of all ESGR resident males. Ages and sizes for successful males were determined from the average of all ages/sizes for the years in which a male was successful over the period of study (1999-2006). Ages for all resident males were adjusted to the age the male in 2003.

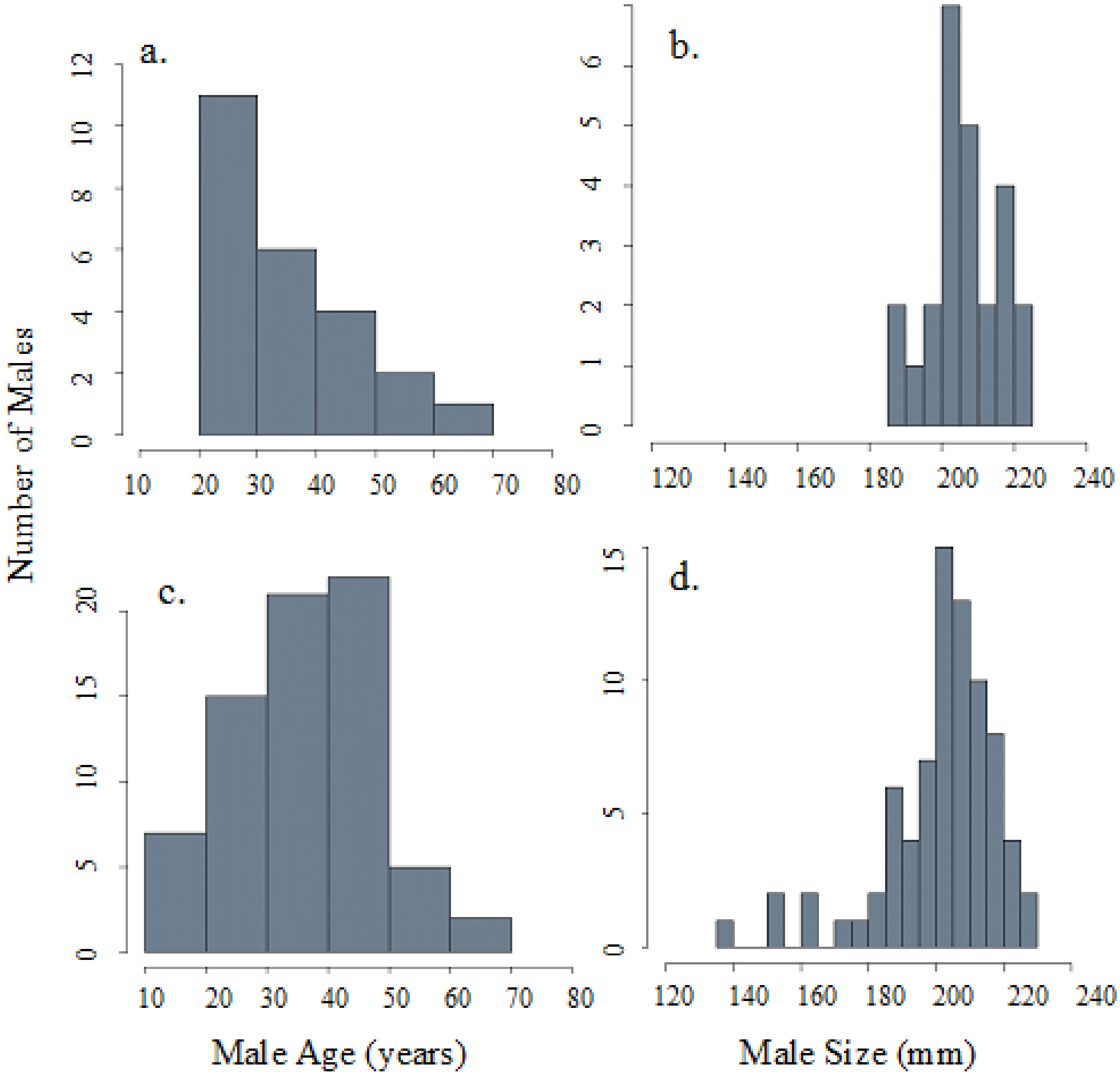


Fig. 3.5. Percentage of nests with evidence of the same male siring offspring with the same female (repeat paternity) as a function of inter-annual nesting interval.

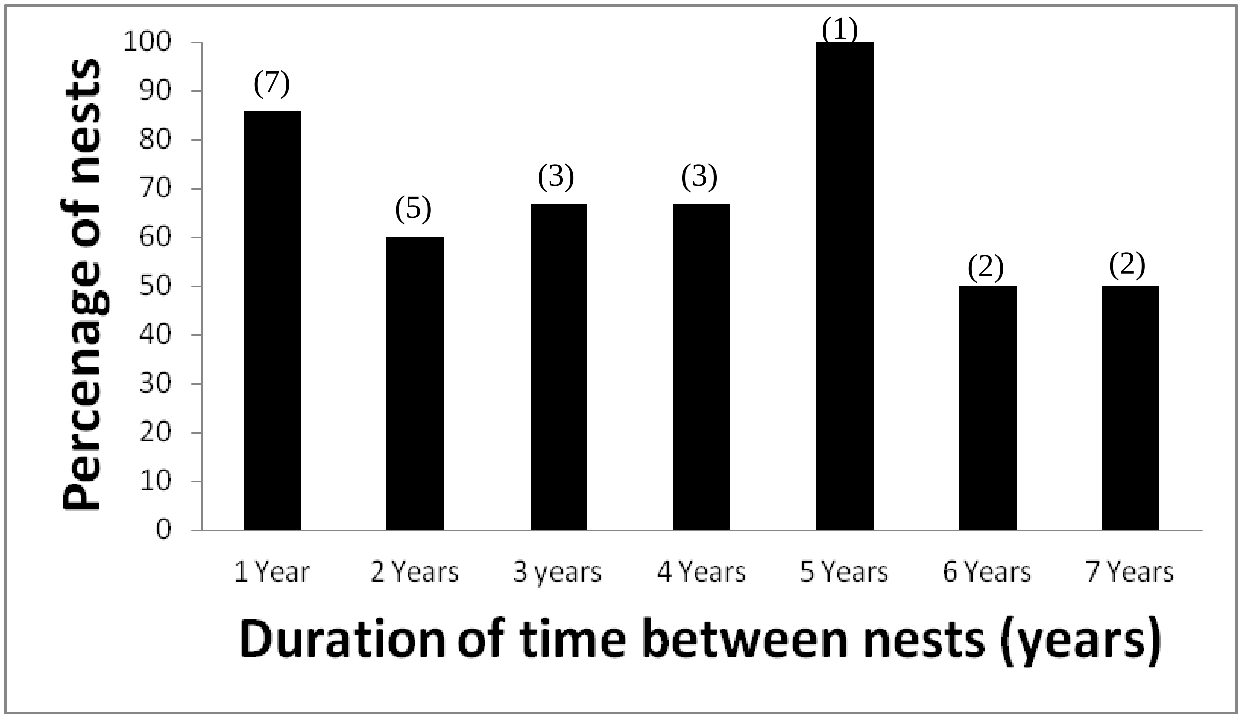
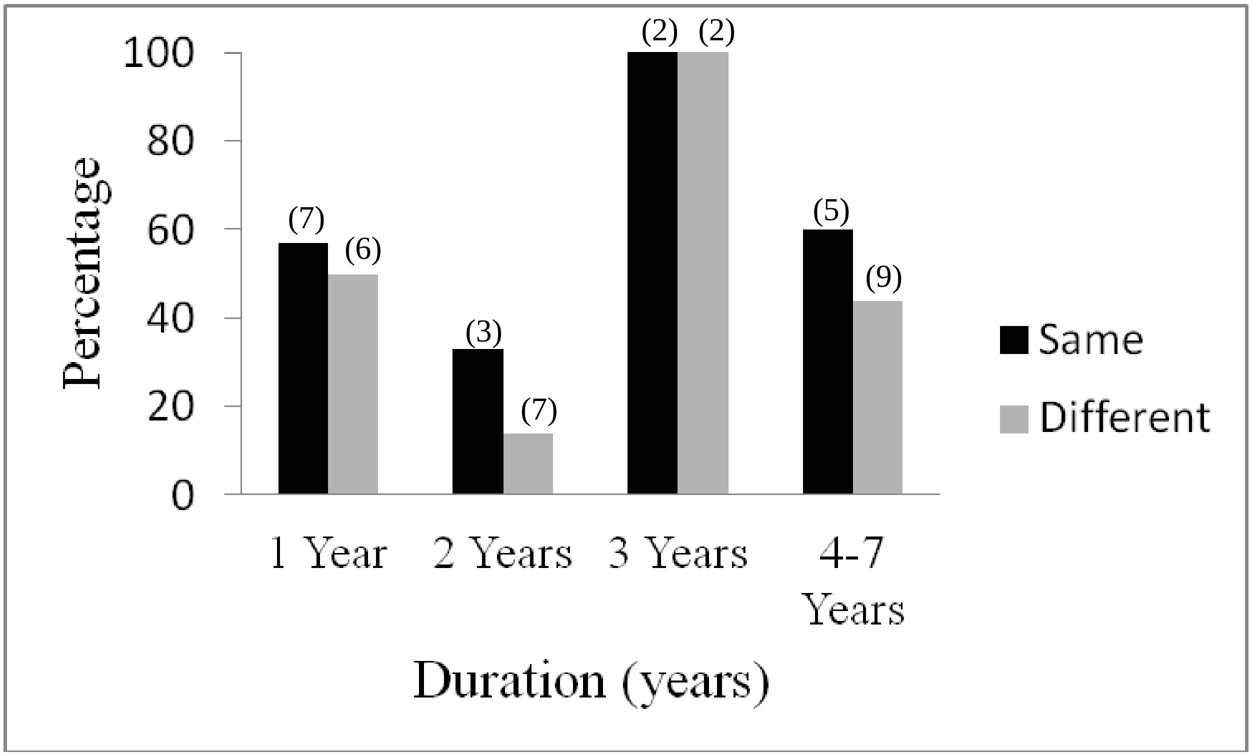


Fig. 3.6. Percentage of among-year comparisons where repeat paternity was observed from males from the same (black) or different (gray) residence wetlands as the female.



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CHAPTER 4: COMPARATIVE ANALYSIS OF FACTORS INFLUENCING MATING SYSTEMS AND MALE REPRODUCTIVE SUCCESS IN FRESHWATER TURTLES

Abstract

Interspecific comparative studies can help to identify factors associated with mating tactics adopted by males and females, particularly when detailed life-history data are available. We used 33 years of reproductive data combined with 4 years of genetic parentage data in three species of freshwater turtles, Painted turtles (*Chrysemys picta*), Blanding's turtle (*Emydoidea blandingii*), and Snapping turtles (*Chelydra serpentina*), to quantify how commonalities and differences in species life history and demography influence multiple paternity, repeat paternity, and male reproductive success. Incidence of multiple paternity was lower among Painted turtles (14.1%) than among Blanding's turtles (46.8%) or Snapping turtles (51.7%). However, incidence of multiple paternity varied among years in all three species. Female age was a significant positive predictor of incidence of multiple paternity in Painted and Snapping turtles. Female body size was a significant positive predictor of incidence of multiple paternity in Blanding's and Snapping turtles. Clutch size was a significant positive predictor of incidence of multiple paternity only in Blanding's turtles. The incidence of females using sperm from the same male(s) to sire offspring among years was 38%, 69.56% , and 66.7% for Painted, Blanding's and Snapping turtles, respectively. Paternity analysis conducted on Painted and Blanding's turtles revealed that the number of clutches sired was a significant positive predictor of male reproductive success in both species. Mate number was a significant positive predictor of male reproductive success in Blanding's turtles but not in Painted turtles. Results demonstrate that variation in reproductive quality among females, particularly frequency of clutch production, significantly and positively affected male reproductive success in Painted and Blanding's turtles, and may be a substantial source of variation in Snapping turtles.

Introduction

Long-term, individually-based reproductive ecology studies allow tests of long held but largely emperically untested predictions in evolutionary ecology (Clutton-Brock et al. 1982; Clutton-Brock 1988). Mating tactics and traits associated with increased reproductive success are most appropriately viewed where the interplay between life history and behavioral ecology allows the relative costs and benefits of such traits to become apparent (Sih and Bell 2008; Bassar et al. 2010). Although life-history studies focus on age- and size-specific survivorship and reproductive rates, traits that enhance competitive mating abilities often do so at the expense of other fitness components, including longevity (Reznick et al. 2000). Thus, traits associated with reproduction often exemplify the potential trade-offs between allocation of resources to current versus future reproductive efforts.

Although the majority of life-history concepts are female based (Stearns 1992), integration of data on males into life-history theory and population dynamics is important (Rankin and Kokko 2007). For example, male density influences fertilization probability (Rankin and Kokko 2007) and rates or intensity of female harassment (Lee and Hays 2004; reviewed in Westneat and Fox 2010); both of which can result in females mating with multiple males (Kokko and Mappes 2005; Sztatecsny et al. 2006). Male density and adult sex ratios can also affect female mate choice, whereby females may become less choosy when males are less frequently encountered (Kokko and Mappes 2005).

Males and females use different tactics to maximize reproductive success (Trivers 1972) despite providing equal genetic contributions to offspring. Variation in reproductive success is primarily a function of resource availability for females (Clutton-Brock et al. 1981; Stearns 1992). Male reproductive success is primarily determined by the number of females mated

(Bateman 1948; Andersson 1994; Rodriguez-Munoz et al. 2010), and by the traits of the females mated (Arnold and Duvall 1994; Owens and Thompson 1994). However for both sexes, variation in demographic and environmental conditions can influence mating behaviors and reproductive success.

Among-year variation in factors associated with reproductive success can result from variation in sex ratio and the number of reproductive individuals (Emlen and Oring 1977; Crowley et al. 1991; Klemme et al. 2007), and within season variation in the ages and traits of the subset of reproductive individuals (Coulson et al. 2001, Dreiss et al. 2010). Demographic variation can alter the number and attributes of potential mates that are encountered and affect the strength of mate preference (Dreiss et al. 2010; Milner et al. 2010). For example, the order in which males are encountered by females and the attributes of those males can influence whether or not individuals will reproduce, and levels of variation in reproductive success of males (e.g. "trading up"; Pitcher et al. 2003; Uller and Olsson 2008).

Reproductive success is typically estimated based on short-term studies that sample a subset of individuals rather than repeated measures of the same individuals over time (Clutton-Brock 1988; Weatherhead and Boag 1997; but see also McElligott and Hayden 2000; fallow bucks; *Dama dama*); Brown et al. 1997, mexican jay; *Aphelcoma ultramarina*). Documentation of reproductive success over multiple reproductive episodes will capture more of the variation experienced over an individual's lifetime and provide a more robust estimate of the factors that influence variation among individuals in reproductive success (Clutton-Brock 1988; Linden and Møller 1989).

Comparisons of mating tactics among species can reveal factors that have shaped life history and behaviors (Tinbergen 1951; Christy and Salmon 1991), and can also provide a basis for developing hypotheses regarding the evolutionary significance of traits (Doughty 1996). However, because mating behaviors are often variable (Magellan and Magurran 2009), and can be influenced by environmental and demographic variation that typically varies over space and time (Emlen and Oring 1977), comparative studies can be enhanced by studying species that cohabit the same area and over the same time period.

Turtles are good subjects for quantifying the relative effects of factors that influence male reproductive success. Turtles provide no post-ovulatory parental care, do not form pair bonds, and are not territorial. Therefore, data on turtle mating tactics may be interpreted in the absence of complex social interactions. However, despite the benefits of studies of less complex systems, less than 5% of behavioral ecology studies have been conducted on taxa other than birds, insects, fish, and mammals (Owens 2006). Long-term studies of reptiles in general and of reproductive success of long-lived, iteroparous species such as turtles specifically are rare (Avise 2001).

For many long-lived organisms, accurate age-specific reproductive information is unavailable due to the inherent difficulties and costs associated with conducting long-term studies (Tinkle 1979). A long-term study on life history and demography of freshwater turtles was initiated by Owen Sexton (1953-1957), and continued by Henry Wilbur (1968-1972, Donald Tinkle (1975-1979), and Justin Congdon (1975-2007) on the E.S. George Reserve (ESGR) in southeastern Michigan. The long-term study was conducted on three species of freshwater turtle (Painted turtles (*Chrysemys picta*), Blanding's turtles (*Emydoidea blandingii*) and Snapping turtles (*Chelydra serpentina*)) that cohabit the same wetland complexes during the same extended time period. Information on reproductive histories of individual females of known ages and sizes, and demographic information such as adult sex ratios, are available for all three species. Additionally, long-term data are available on variation in female reproductive quality (i.e., age- and size-specific clutch size, clutch frequency, and egg size). Populations of all three species on the ESGR have been essentially stable over the past 3 decades. Data from those studies provide an opportunity to use information on demography, life history, and female reproduction as a basis for conducting a shorter term genetic study of factors influencing the mating system and male reproductive success

The goals of this study were to characterize and compare life-history, demographic, and genetic information of turtles on the ESGR to test predictions of how variation among species in life history and demography influence differences in 1) incidence of multiple paternity, 2) incidence of repeat paternity and 3) male reproductive success. There are substantial differences in population sizes and sex ratios, life-history trait values, and behavior among the three species on the ESGR (Table 4.1). In the sections below, we summarize demographic, life-history , and behavioral characteristics traits of the three species of turtles and provide information on reproductive qualities of females (Table 4.1). We propose several factors that can directly or indirectly influence the incidence of multiple or repeat paternity in Table 4.2 and suggest predictions of higher or lower values of incidence of multiple or repeat paternity likely to be exhibited by each species for trait values described. For example, based solely on differences in sex ratio, we predict that multiple paternity will be higher in Painted turtles compared to Blanding's or Snapping turtles because there are more than twice the number of adult males than females in the ESGR Painted turtle population.

Predictions outlined in Table 4.2 for multiple and repeat paternity, assume that all other traits are held constant. Traits that influence male reproductive success are not usually independent (Fig. 4.2), and the patterns observed in multiple paternity, repeat paternity, and reproductive success represent the outcomes of several processes (e.g. pre- and post-copulatory mechanisms; Uller and Olsson 2008). We integrate the results from each individual prediction (Table 4.2) and through comparisons within and among species, explain how factors interact to influence male reproductive success (Fig. 4.2).

Sources of Variation in Female Quality

Variation in Clutch Size and Egg size

Within species variation in clutch size (number of eggs produced per reproductive bout) can affect male reproductive success (number of offspring). In Painted turtles, average clutch sizes are smallest (6.99, *SD* = 0.19) and are similar between the first (mean = 6.9, *SD* = 1.47) and second (mean = 6.7, *SD* = 1.55) clutches produced within a season. Clutch sizes of Blanding's turtles average 9.67 (*SD* = 0.40) and Snapping turtles clutch sizes average 20.80 (*SD* = 1.05). Clutch size is positively related to females body size in all three species (Tables 4.1 and 4.3).

Although most female turtles do not provide behavioral or trophic parental care, they do provide hatchlings with substantial yolk reserves necessary to sustain juveniles during early post-hatchling periods (Congdon et al. 1983). Because egg widths are positively related to egg size, yolk volume and hatchling size, variation in egg widths within a species represent another measure of offspring (and thus female) quality. Egg widths average 17.3 mm (*SD* = 0.22) in Painted turtles, 23.7 mm (*SD* = 0.16) in Blanding's turtle, and 27.6 mm (*SD* = 0.29) in Snapping turtles. In Painted and Snapping turtles, egg widths from X-radiographs were positively correlated with female body size and age (Tables 4.1 and 4.3), whereas in Blanding’s turtles egg widths were not strongly correlated with female body size or age.

The strong positive relationship between body size and egg size in a small-bodied species such as Painted turtles is in part due to relaxation of constraints on egg width caused by the size of the pelvic aperture (Tucker et al. 1978; Congdon and Gibbons 1987), but is not known to occur in larger-bodied species, such as Snapping turtles. Larger Painted turtles produce larger eggs, suggesting that increasing offspring quality outweighs the cost of producing fewer offspring (Smith and Fretwell 1974). Because there are positive relationships between body size and clutch size and egg size in Painted and Snapping turtles (Tables 4.1 and 4.3), variation in female body size provides males with a reliable indicator of female quality.

Clutch Frequency

Average reproductive frequencies were 1.3, 0.80, and 0.85 for Painted, Blanding's, and Snapping turtles, respectively (Painted turtles can produce more than one clutch per year; Table 4.1), and among year reproductive frequencies vary among females of all three species (Table 4.1). Although Blanding's and Snapping turtles produce a maximum of one clutch per year and Painted turtles can produce up to 2 clutches per year (Congdon et al., 2001, 2003), some females of all three species fail to reproduce each year (Table 4.1).

Reproductive frequency is an important component of female reproductive output (i.e., number of offspring produced = clutch size * number of clutches) and the number of offspring a male sires with a female is how male reproductive success is achieved. Therefore, production of second clutches within a year by Painted turtle females coupled with a high frequency of repeat paternity in those clutches represents a major way for males to increase reproductive success that is not available to Blanding’s and Snapping turtle males. Gist and Congdon (1998) hypothesized that a primary function of stored sperm is to fertilize sequential clutches within a year due to the short period (less than 6 days) between clutches when eggs in oviducts of females can be fertilized before shelling of eggs begins. The Gist and Congdon hypothesis was supported in

ESGR painted turtles where repeat paternity was observed in >95% of second clutches (McGuire et al. 2011) and was observed in > 85% of clutches in a Painted turtle population in Illinois (Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002).

Female turtles store sperm (Ewing 1943; Gist and Jones 1989), and repeat paternity has been documented within and among years in several turtle species (Fitzsimmons 1998; Kichler et al. 1999; Pearse and Avise 2001; Pearse et al. 2001; Pearse et al. 2002; Roques et al. 2006; Lee 2008; McGuire et al. 2011). Therefore, we predict that repeat paternity among years will be observed in all three species (Table 4.2) and the potential benefits to male reproductive success will be substantial. Longer durations between reproductive intervals can increase the likelihood of individuals encountering and mating with different individuals. Additionally, declines in sperm viability or sperm depletion (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998) may decrease the likelihood of stored sperm use over longer time intervals. Increases in encounters among years and multiple matings (Uller and Olsson 2008) could increase incidence of multiple paternity (Table 4.2).

Female body size and age are positively associated with female quality (clutch size, clutch frequency, and egg size; Congdon et al. 2001; Congdon et al. 2003). Males can obtain reproductive benefits by mating with high-quality females. Therefore, the number of males attempting to mate and the intensity of courtship may increase with larger and older females. Therefore we predict that in all three species, incidence of multiple paternity will be higher in clutches from older/larger females than in clutches from younger/smaller females (Table 4.2). Increased encounters with males may decrease the incidence of repeat paternity in older/larger females (Table 4.2).

Source of variation in number of mates

Courtship

Courtship behaviors can influence mate number by providing an opportunity for mate choice (Halliday 1983; Wiley and Poston 1996). Male courtship in Painted turtles involves use of the foreclaws to titillate the face of the female (Berry and Shine 1980; Harding 1997). Male courtship in Blanding's turtles involves chasing, swaying, chin inflation, head chinning, head weaving, shell thumping, gulping, and bubbling (Graham and Doyle 1979; Baker and Gillingham 1983; Ernst et al. 1994). Snapping turtle mating includes face to face posturing with head swinging (Legler 1955) and water gulping, bubbling, and biting of the head and neck of the female (Taylor 1933; Ernst et al. 1994). Although courtship behaviors allow individuals to evaluate potential mates, to date there are no studies that document mate choice in the Painted, Blanding's or Snapping turtles.

Sexual Size Dimorphism

Sexual size dimorphism can affect courtship and mating behaviors (Berry and Shine 1980; Weatherhead et al. 1995; Fairbairn 1997; Zamudio 1998; Blanckenhorn 2005). Among the species on the ESGR all three possible size combinations occur: 1) females are larger than males in Painted turtles (Congdon et al. 2003), 2) male and females are similar in size in Blanding's turtles (Graham and Doyle 1979; Congdon and van Loben Sels 1991; Rowe 1992; Germano et al. 2000; Pappas et al. 2000), and 3) males are substantially larger than females in Snapping turtles (Berry and Shine 1980; Congdon et al. 2008; Table 4.1). The larger size of Snapping turtle males contributed to speculation that males force copulations (Berry and Shine 1980). If Snapping turtle males are able to forcefully copulate with females, the incidence of multiple paternity may be higher than in other species (Table 4.2; Berry and Shine, 1980). However, because only a subset of males would be large enough to force copulations, we predict that more multiple matings may occur in smaller females and small males should not be able to mate with large females (Table 4.2).

Population sizes and sex ratios

Population size is positively associated with multiple paternity among some sea turtle populations (Ireland et al. 2003; Jensen et al. 2006). If freshwater turtles exhibit similar tendencies, we predict that multiple paternity will be higher in Painted turtles (largest population) than in Blanding's or Snapping turtles (both smaller populations; Table 4.2). Adult sex ratios are male biased (2.3:1) in Painted turtles, and approximately 1:1 in resident Blanding’s (Congdon et al. 2011) and Snapping turtles (Congdon et al 1987). Based on differences in adult sex ratios (Table 4.1), we predict that multiple paternity will be higher in Painted turtles than in Blanding’s and Snapping turtles (Table 4.2). However, because females would encounter more males (and thus more opportunities to mate with different individuals), repeat paternity will be lower in Painted turtles than Blanding's or Snapping turtles with respect to both population size and sex ratio (Table 4.2).

If all males are reproductive each year, variation in the number of reproductive females each year will influence the operational sex ratio (Fig 4.1). Mating attempts among the females that are reproductive may increase in years when the number of reproductive females is low. We predict that incidence of multiple paternity will increase and repeat paternity will decrease in years where the number of reproductive females is low (Fig. 4.1, Table 4.2)

Morphological traits that influence terrestrial movement

The three species have different shell characteristics that influence the degree to which individuals use terrestrial habitats, that can in turn influence mate encounter rates. The shells of Blanding's turtles are hinged and provide protection from predators and reduce desiccation (Greene 1988; Ernst and Barbour 1989; Martin et al. 2005) while they are in terrestrial habitats. The large body size and aggressive behavior of Snapping turtles provides some protection from some predators. However their reduced plastron and skin characteristics increases susceptibility to desiccation while in terrestrial habitats (Finkler 2001). Painted turtles are the smallest of the 3 species and their body size combined with rudimentary kinesis of the plastron provides little protection from predators and nesting females experience much higher predation rates than do Blanding’s or Snapping turtles.

Terrestrial movements of Painted and Snapping turtles are infrequent except for female nesting migrations that are short in distance and duration compared to those of Blanding’s turtles. Male and female Blanding's turtles make frequent terrestrial movements to ephemeral wetlands, and females make nesting migrations up to 1600m (Congdon et al. 2011). Painted and Snapping turtle females make much shorter nesting migrations (an average of 65.9 and 34.5m respectively). Unlike the hatchlings of Blanding's turtles, hatchlings of both Painted and Snapping turtles have a high probability dispersing from nests directly into the residence wetland of their parents. If matings occur in residence and ephemeral wetlands, then the number of potential mates encountered in both habitats will probably be larger than just those encountered within their residence wetland (Table 4.2). Additionally, if individuals are more widely distributed across the landscape, mate density may decrease, which can affect mate choice and mate number (Table 4.2).

Methods

Field Methods

The field methods used to collect size, age, and reproductive data for females of all three species of turtles on the ESGR were essentially the same with two substantial exceptions. First, sample collection of Painted turtles was restricted to East Marsh (and surrounding nesting areas) because the population size of Painted turtles is substantially larger than those of Blanding's turtles or Snapping turtles. Blanding's turtles and Snapping turtles were sampled from all areas on the ESGR. Second, the use of a drift fence that completely surrounded East Marsh was not equally effective at capturing females of all species. Whereas nearly all gravid Painted turtle females were captured at the fence as they moved to nesting areas during daylight hours, some Snapping turtle females nested in the early morning (between 0100-0500 h) when the drift fence was not monitored consistently in most years. The use of the drift fence was least effective for Blanding’s turtles because some females move to ephemeral wetlands before the beginning of the nesting season.

Reproductive frequency

To compare inter-annual reproductive frequency among the three species, we excluded among-year intervals > 4 years because long intervals probably represent unusual durations between reproductive events (e.g. those resulting from injuries, disease, temporary movements to other wetlands, and nesting off of the ESGR). Because Painted turtles can produce two clutches in a year, whereas Blanding's and Snapping turtles produce a maximum of one, we restricted reproductive frequency to only among-year reproductive events. We calculated the mean reproductive intervals of individuals to standardize variation in total inter-capture intervals ranging from 5 to >30 years.

Genetic Methods

Snapping turtle DNA was extracted from blood (adults; N = 461) or tissue (hatchlings; N = 89 nests, 1575 hatchlings) using the Qiagen DNeasy kit (Qiagen Inc, Valencia, CA) and amplified 4 microsatellite loci Cp3, (Pearse et al. 2001), BTCA7, BTGA5 (Libants et al. 2004), and D111 (Hackler et al. 2007). Painted turtle samples (N = 535 adults, 155 nests, 1054 hatchlings) were genotyped at 6 microsatellite loci (Cp2, Cp3, (Pearse et al. 2001), BTGA2, BTGA3, BTCA7, (Libants et al. 2004) and EB11 (Osentoski et al. 2002)). Blanding's turtle samples (247 adults, 77 nests, 611 hatchlings) were genotyped at 8 microsatellite loci including BTGA5, BTCA11, BTCA9, (Libants et al. 2004), EB11, EB19, EB17, EB09 (Osentoski et al. 2002), and GmuD70 (King and Julian 2004).

For all three species, amplification of DNA was performed using polymerase chain reaction (PCR) using published conditions and separated using gel electrophoresis on 6% polyacrylamide gels. We used an FMBIOII scanner (Hitachi Inc., Tokyo, Japan) to visualize PCR products. All genotypes were independently scored by a minimum of two experienced laboratory personnel. To estimate error, and ensure genotyping consistency, 10% of adults were randomly selected and re-genotyped for all loci. Estimates of expected heterozygosity, number of alleles, the exclusion probability with one parent known, and tests for Hardy-Weinberg Equilibrium were performed using the program CERVUS 3.1.0 (Kalinowski et al. 2007).

Paternity, multiple paternity, and repeat paternity

For Painted and Blanding's turtles, we utilized the methods for parentage, multiple paternity, and repeated paternity as described in Chapters 2 and 3 that relied on consensus between two parentage programs: NEST (Jones et al. 2007) and CERVUS (Kalinowski et al. 2007).

For Snapping turtles, we used a minimum allele count to determine whether more than one male sired offspring within a clutch (multiple paternity). Because we observed females in nesting activity, we could identify paternal alleles. When 3 or more paternal alleles at a single locus could be identified, the clutch was assigned as multiple paternity.

Paternal alleles were used to reconstruct multi-locus paternal genotypes that were compared among years to provide evidence of repeat paternity. When reconstructed paternal genotypes were consistent among years at all loci, the clutch was identified as repeat paternity. Estimates of repeat paternity were based on all available data for all three species (Painted turtles 2003-2006, Blanding's turtles 1999-2006, Snapping turtles 2002-2006).

We lacked sufficient power to determine paternity for Snapping turtles and therefore analyses of male reproductive success were restricted to Painted turtles and Blanding's turtles.

Statistical Analyses

We quantified associations between female age, female body size (carapace length), clutch size (number of eggs), with incidence of multiple paternity for Painted turtles, Blanding's turtles, and Snapping turtles using generalized linear mixed models (GLMM) using multiple paternity as a binomial dependent variable. Female was used as a random effect in all models because nests from the same female were often sampled more than once in the course of the study. Data for all three species were primarily based on samples collected from 2003-2006 to facilitate comparisons among the species.

We tested for associations between female age and body size with incidence of multiple paternity for Painted turtles and Snapping turtles using generalized linear mixed models (GLMM) with repeat paternity as a binomial dependent variable. Low sample sizes precluded our ability to use a mixed model in Blanding's turtles and so models were run as generalized linear models (GLM) with repeat paternity as a binomial dependent variable.

To quantify associations between the total number of mates, the total number of clutches, the average offspring per clutch, and whether or not a clutch had multiple sires (multiple paternity) with male reproductive success (total number of offspring sired) using general linear models (GLM). Mixed model analysis was not necessary because each male was only represented once in the dataset. We used average values for the number of offspring per clutch and multiple paternity determined for each male.

Results

Variation in female numbers and quality

The number of reproductive females of all three species varied among years (Fig. 4.1a). However, the annual number of reproductive females was not correlated among the species (Pearson; $\rho < 0.299$, df = 23, $P > 0.156$).

The average carapace lengths of female Painted and Blanding's turtles increased slightly over the course of the life history study; whereas the average carapace length of Snapping turtle females decreased (Fig. 4.1b). Because all three species of turtles are long-lived (i.e., some individuals remained in the study over many years) it was expected that the average ages of all three species would increase over time (Fig. 4.1c).

Carapace length of females was significantly and positively related to age in all three species, but the relationship was strongest in Snapping turtles ($R^2 = 0.59$) and Painted turtles ($R^2 = 0.33$) compared to Blanding's turtle ($R^2 = 0.10$; Table 4.1). The relationships between clutch size and body size and clutch size and age were similar among species (Table 4.1). Egg widths increased significantly with body size and age of females in Painted and snapping turtles, but those relationships were weak in Blanding's turtles (Table 4.1).

Average among-year reproduction intervals were slightly shorter for Painted turtles than for Blanding's or Snapping turtles (Painted turtles; mean = 1.5, min-max = 1-4, *SD* = 0.43; Blanding's turtles, mean = 1.7, min-max = 1-4, *SD* = 0.55); Snapping turtles, mean = 1.7, min-max = 1-3, *SD* = 0.49; Table 4.4).

Analyses based on genetic data

Genetic diversity

For all three species, all loci were in Hardy-Weinberg Equilibrium (HWE; $P > 0.10$) and loci were independent ($P > 0.10$). In Painted turtles, the mean number of alleles per locus over the six loci was 19.2 (min-max across loci = 7-34), mean expected heterozygosity was 0.807 (min-max across loci = 0.689-0.885), and the multi-locus exclusion probability with one parent known was 0.999. In Blanding's turtles, loci were polymorphic with an average of 7.4 alleles (min-max = 4 - 15 alleles) and a multi-locus estimate of expected heterozygosity of 0.69 (min-max = 0.54 - 0.85). The multi-locus non-exclusion probability with one parent known was

0.996. In Snapping turtles, the mean number of alleles per locus was 17.8 (min-max = 4-27) and the mean expected heterozygosity was 0.68 (min-max = 0.15 - 0.91). For Snapping turtles, the multi-locus exclusion probability with one parent known was 0.984. Insufficient power precluded paternity analysis. For all three species the empirically estimated multi-locus error rate was less than 1% (mean over all loci).

Multiple paternity

Over four years (2003-2006), the incidence of multiple paternity averaged 14.1%, 46.8%, 51.7% for Painted turtles, Blanding's turtles, and Snapping turtles, respectively. Annual proportions of multiple paternity within species also varied among years for Painted turtles (30.0, 12.5, 14.3 and 6.1%), Blanding's turtles (53.8, 55.6, 36.4, and 42.9%), and Snapping turtles (61.5, 57.1, 44.4, and 50.0%) for 2003-2006, respectively.

The three species varied in the relationships between the number of reproductive females and incidence of multiple paternity. In Painted turtles, the number of reproductive females was inversely related to the incidence of multiple paternity among high-quality females (McGuire et al. 2011). However the relationship between multiple paternity and the number of reproductive female Painted turtles was less pronounced among all females (Chapter 2). In Blanding's turtles, from 2003-2006 there was no substantial variation in the total number of reproductive females (includes both residents and non-residents; Chapter 3). However, among all years of genetic sampling for Blanding's turtles (1999-2006), the year with the lowest multiple paternity, also had the highest number of reproductive females. In Snapping turtles, only one year (2004) had a low number of reproductive females and multiple paternity was 57.1%, which was consistent with the average amount of multiple paternity for the species.

The three species exhibited similarities in the attributes associated with incidence of multiple paternity. In Painted turtles, female age was a significant predictor of multiple paternity (Table 4.5; GLMM; age, $Z = 2.01$, $P = 0.044$) whereas female body size and clutch size were not (body size, $Z = -0.228$, $P = 0.819$; clutch size $Z = -0.316$, $P = 0.752$). Age of Blanding's turtle females was not a significant predictor of multiple paternity (Table 4.5; GLMM; $Z = 0.977$, $P = 0.328$), but body size was marginally significant (GLMM; $Z = 1.896$, $P = 0.058$) and clutch size was a significant predictor of multiple paternity (GLMM; $Z = 2.507$, $P = 0.012$). However, when the entire dataset is used for Blanding's turtles (1999-2003), female age, body size, and clutch size were all significant (GLMM; age, $Z = 2.04$, $P = 0.042$; body size $Z = 2.19$, $P = 0.029$, clutch size $Z = 2.787$, $P = 0.005$). In Snapping turtles, female age and body size (carapace length) were both significant predictors of multiple paternity (Table 4.5; GLMM; age, $Z = 2.67$, $P = 0.008$; body size, $Z = 2.62$, $P = 0.009$), whereas clutch size was not (GLMM; $Z = 0.92$, $P = 0.357$).

Repeat paternity

Paternal genotypes that were consistent with the same male(s) siring offspring with the same female among years (repeat paternity) was observed in each species. The mean incidence of repeat paternity was (38%, 69.56%, and 66.7% for Painted, Blanding's and Snapping turtles respectively; Fig. 4.3). In two of the three species, the incidence of repeat paternity was higher in clutches produced in consecutive years than for longer inter-clutch intervals (38.0%, 85.7%, and 82.4% for Painted, Blanding's and Snapping turtles respectively; Fig. 4.3). In all three species no female attributes (age, size) were associated with incidence of repeat paternity (Painted turtle; GLMM; age, $Z = 0.043$; $P = 0.966$; size, $Z = 0.792$, $P = 0.429$; Blanding's turtle;

GLM (GLMM did not converge); age, $Z = 0.958$, $P = 0.338$; size, $Z = 0.698$, $P = 0.485$; Snapping turtle; GLMM; age, $Z = 1.464$, $P = 0.143$, size Z = 1.44, $P = 0.150$).

Male reproductive success (total number of offspring)

In Painted turtles, male reproductive success was significantly associated with the number of clutches sired (Table 4.6; GLM, $t = 10.238$; $P < 0.001$) and the average number of offspring sired per clutch (GLM, $t = 4.252$, $P < 0.001$). Male reproductive success was not associated with the number of mates (Table 4.6; GLM, $t = 1.181$; $P = 0.242$) or attributes of the male (GLM; body size; $t = 0.872$; $P = 0.387$; age: $t = -1.132$, $P = 0.272$) and was marginally associated with multiple paternity (GLM; $t = -1.90$; $P = 0.06$).

Male reproductive success in Blanding's turtles was influenced by the total number of mates, the total number of clutches, and whether the clutch was sired by more than one male (Table 4.6; GLM; total mates, $t = 4.355$, $P <0.001$; total clutches, $t = 8.071$, $P <0.001$; multiple paternity, $t = -2.348$, $P = 0.027$). Male reproductive success was not influenced by the average number of offspring sired per clutch (Table 4.6; GLM, $t = 1.732$, $P = 0.096$) or attributes of the male (Table 4.6; GLM; body size, $t = 0.794$, $P = 0.437$; age, $t = 0.084$, $P = 0.934$).

Discussion

Variation in female reproduction

The number of reproductive females varied substantially among-years in all three species (1983-2006; Fig. 4.1a). However, annually the numbers of reproductive females each year were not correlated among species, suggesting that annual resource abundance was not the only factor involved and that dietary differences among species may play a role in determining the number of reproductive females each year. Although average body sizes and ages of Painted turtles and Blanding's turtles increased over the duration of the life history study, the average body size of female Snapping turtles declined from the mid-1990s through 2006 while ages generally increased (Fig. 1b,c). The decline in body size apparently resulted from smaller primiparous females recruiting into the reproductive population. Continued recruitment of small primiparous females into the Snapping turtle reproductive population made up of larger females could strengthen the relationship between body size and age. However, the relationship between body size and age was essentially stable over the entire period (prior to 1991 $R^2 = 0.56$ and from 1983-2006 ($R^2 = 0.59$; Table 4.1). Therefore relationships between body size and age seem to be unaffected by the recruitment of smaller reproductive females, but overall among female variation in body size and reproductive quality would still increase.

Female reproduction is typically limited by the resource availability (Stearns 1992) and reproductive intervals were variable among females within each species. Painted turtles on the ESGR produce a maximum of two clutches per year while Blanding's and Snapping turtle females produce a maximum of one clutch annually, however, in all three species some females fail to reproduce every year (Congdon et al. 2001; Congdon et al. 2003). Even though Painted turtle females produce second clutches in some years, inter-annual reproductive intervals were similar to those of female Blanding's and Snapping turtles (Table 4.4). Producing a second clutch of eggs represents a substantial reproductive investment that can delay the time until the next reproductive event for Painted turtles. However, the females with a high propensity to produce two clutches actually had shorter among-year reproductive intervals (Chapter 2) than did female Painted turtles that produced only one clutch. Reproductive allocation to the single clutch averaging 10 and 28 eggs is substantially different for Blanding's turtles and Snapping turtles, respectively. (Table 4.1). However, the intervals between reproductive events were similar (reproductive intervals = 1.66 for both Blanding's and Snapping turtles; Table 4.4). Despite the production of second clutches by Painted turtles, and the substantial difference in the size of the single cutch produced by Blanding's and Snapping turtles, similar reproductive intervals of all three species appear to be possible due to the resource-rich environment of ESGR wetlands (Fiala and Congdon 1983). However, among female variation in reproductive frequencies within species remains a potentially important source of variation in male reproductive success.

Multiple paternity

Within-species variation in the number of reproductive females influenced the incidence of multiple paternity. Incidence of multiple paternity was inversely related to the number of reproductive females among high-quality females in Painted turtles (McGuire et al. 2011), and among all reproductive females (resident and non-residents) in Blanding's turtles (Chapter 3). However, there was no relationship between the number of reproductive females and multiple paternity in Snapping turtles, and the relationship was weak between multiple paternity and the number of reproductive females in Painted turtles when females of all reproductive qualities were considered. If the number of reproductive males remains constant, variation in the number of reproductive females among years will change the operation sex ratio. For example, if the number of reproductive females declines, the operational sex ratio becomes more male biased, and more males can result in more attempts to mate with each female. In Blanding's turtles, the number of reproductive females was inversely associated with the annual proportion of clutches with multiple paternity (Chapter 3). In Painted turtles, the proportions of clutches exhibiting multiple paternity was positively related to ages of females in years where the number of reproductive females was low, but not when the number of reproductive females was high (Chapter 2).

Several direct and indirect benefits may exist for males and females that have more than one mate (Zeh and Zeh 1996). Aside from fertilization assurance, direct benefits are unlikely because turtles provide no post-ovulatory parental care and no trophic resources are provided by males prior to or following matings (Uller and Olsson 2008). Females may obtain indirect benefits (e.g. "good genes") through pre-copulatory mate choice or post-copulatory sexual selection (e.g. sperm competition or cryptic female choice) that can facilitate genetic compatibility (Zeh and Zeh 1996; Uller and Olsson 2008). However, to date no empirical evidence supports that female turtles benefit from polyandry (Lee and Hays 2004). An alternative hypothesis for multiple matings is "convenience polyandry" (Thornhill and Alcock 1983), where females are expected to resist repeat matings unless the cost of resisting a male exceeds the cost of mating.

In turtles, costs of matings for females could result from male harassment or through the potential for forced copulation in situations where males are larger than the females. Harassment of females has been shown to increase multiple matings in taxa other than turtles (water striders *(Aquarius remigis),* Watson et al. 1998; common toad (*Bufo bufo*); Sztatecsny et al. 2006) and has been proposed in sea-turtles (Green turtles; *Chelonia mydas*; Lee and Hays 2004). The male-biased adult sex ratio in the ESGR population of Painted turtles could result in female harassment, and high levels of multiple paternity, particularly in years when low numbers of reproductive females reproduce. However, Painted turtles had the lowest incidence of multiple paternity of the three species, suggesting that if harassment increases multiple matings, additional mechanisms (e.g. post-copulatory sexual selection) may also exist. Sexual size dimorphism where males are larger occurs in Snapping turtles and forced copulation has been suggested as a male mating tactic (Berry and Shine 1980). However, because size ranges of males and females overlap, the size differences of only a subset of mating pairs would be sufficiently large enough to make forced copulations probable. Thus if forced copulations is a mechanism leading to females mating multiply, the highest incidence of multiple matings should occur in smaller females. In contrast, we observed that multiple paternity occurred most frequently in the largest females; a pattern suggesting that increased levels of multiple paternity occur as a function of males seeking copulations with females of high reproductive quality.

Female body size represents one reliable trait that males may use to identify females of higher reproductive quality because body size was positively correlated with clutch size in all three species (Tinkle et al. 1981; Congdon et al. 1987; Congdon and van Loben Sels 1991). However, to date, there is no behavioral evidence for male preference for larger females. Clutch size was positively associated with incidence of multiple paternity in Blanding's turtles and Snapping turtles, but was not associated with multiple paternity in Painted turtles (Table 4.5). In Snapping turtles, body size was a significant predictor of multiple paternity but clutch size was not, suggesting that there may be higher competition for larger, more fecund, females compared to smaller females, and that the effect of body size on multiple paternity is not a function of detection bias.

In the four years that genetic data were collected we did not observe an association between female age and multiple paternity in Blanding's turtles (Table 4.5). However, when samples from a previous study on the ESGR were included (Osentoski 2002), female age was significantly and positively associated with incidence of multiple paternity (Chapter 3). Therefore, female age was associated with incidence of multiple paternity of clutches of all three species. Because female body size and age is positively related, males may use a reliable trait to identify older and high quality females. Female reproductive frequency has been shown to increase with age in all three species (Blanding's turtle, Congdon et al. 2001; Painted turtle, Congdon et al. 2003; Snapping turtle J.D. Congdon unpublished data). The positive relationship between the incidence of multiple paternity and female age in all three species suggests that males can identify body size or some other phenotypic traits to identify high-quality older females.

Males assess mate quality when investing resources toward pre-copulatory mating tactics (Bonduriansky 2001; Craig et al. 2002; Zhang et al. 2010). In desert gobies (*Chlamydogobius eremius*), males associated with larger (more fecund) females for longer periods than they did with smaller females, and males increased signaling efforts when presented with a larger females compared to smaller females (Wong and Svensson 2009). In Tibetan macaques (*Macaca thibetana*), males spent more time associating with high-quality females and more males were attracted to high-quality females than to lower-quality females (Zhang et al. 2010). Although there is no evidence of social structure or pair bonding in turtles, nor direct behavioral evidence of preferences, that multiple paternity increases among older and larger females suggests that males may be able to identify and attempt to mate with high-quality females.

Male reproductive success

Multiple paternity was negatively associated with male reproductive success in Blanding's turtles and was marginally (and negatively) significant in Painted turtles (Table 4.6). Among successful males, multiple paternity will have an overall negative effect on male reproductive success because multiple paternity results in sharing of paternity within a clutch. The average number of offspring sired per clutch was a significant source of variation in male reproductive success in Painted turtles, but was only marginally significant in Blanding's turtles (Table 4.6). Male reproductive success was positively associated with the number of clutches a male sired in Painted and Blanding's turtles (Table 4.6). However, the means by which males were able to increase the number of clutches differed between Painted and Blanding's turtles. The number of mates was a significant predictor in Blanding's turtles, but was not significant in Painted turtles. In contrast, in Painted turtles the vast majority of males sired offspring with only a single female. Males increased the number of clutches sired without increasing the number of mates through repeat paternity with the same female within and among years. Blanding's turtle males, however, increased the number of clutches sired through repeat paternity or by mating with multiple females.

The time between production of clutches exhibiting repeat paternity was limited only by the duration of the study. Repeat paternity extending three and more years was common in all three species (Fig. 4.3) and could have resulted from remating with the same females, or from the use of stored sperm by females. For Painted turtles, repeat paternity between clutches within a year is probably the result of stored sperm use due to the short window of receptivity between clutches (Gist and Congdon 1998) since repeat paternity was observed nearly 100% of the time between clutches within a year (Fig. 4.3). Among years, opportunity for repeat matings increases, and the likelihood of a female using stored sperm use decreases with the time elapsed between nesting events. In addition, several studies have shown declines in fertility over time when females are isolated from males, which could be the result of a decline in sperm viability or sperm depletion (Goin et al. 1978; Gist and Jones 1987; Palmer et al. 1998). However, during the present study, a decline in the number of fertilized eggs was not observed in clutches of all three species. The same males sired all eggs in clutches with 4 and 7 years intervals in Painted turtles and Blanding's turtles, respectively. No attributes of the female were associated with repeat paternity in any species, therefore no subsets of females showed a greater propensity to have the same male sire clutches among years. In combination, the relatively low numbers of mates of both males and females, the frequency of occurrence of repeat paternity (especially for Painted turtles), and the extended among-year inter-clutch intervals between repeat paternity raise questions about the current view of turtle mating systems and behavioral biology that warrants further explanation. Future work should focus on detailed behavioral observations and experiments designed to disentangle remating from stored sperm mechanisms of repeat paternity and attempt to answer the following questions: why aren't some females mating with different males among years if males are plentiful; and, why aren't male Painted turtles successfully mating with more than one female? Despite differences in the life history, demography, and behavior of the three species (Table 4.1) we found commonalities in the attributes associated with multiple paternity and the determinants of reproductive success. For all three species, multiple paternity increased with female age and long-durations of repeated paternity were observed. In Painted and Blanding's turtles, multiple paternity negatively influenced male reproductive success. Thus males that mated with older females would share paternity more often than males that mate with younger females. Female age was positively associated with clutch frequency in all three species (Blanding's turtle, Congdon et al. 2001; Painted turtle, Congdon et al. 2003; Snapping turtle J.D. Congdon unpublished data). In all three species, increased clutch frequency among older females, combined with the high levels of repeat paternity, suggests that, despite sharing paternity, the benefits of siring multiple clutches of older females among years can substantially increase male reproductive success. The number of clutches that a male sired was positively associated with reproductive success in both Painted and Blanding's turtles. Although we could not test attributes associated with success in Snapping turtles, because we observed high levels of repeat paternity among clutches, we predict the number of clutches would also be a substantial determinant of male reproductive success in Snapping turtles. Because body size is positively correlated with age in all three species, males may be able to use size as a reliable indicator of female age. However, in Painted turtles, age but not body size was a positive predictor of multiple paternity. Thus, males may also rely on additional cues to identify older females.

Older females represent a valuable resource for populations both in terms of increased reproductive output as well as for maintaining genetic diversity. Clutches sired by more than one male can reduce levels of cohort-specific coancestry. Consistent age-specific patterns among the three species with respect to clutch frequency and multiple paternity demonstrate the importance of older females to populations. Conservation plans should include ways to maintain high survivorships of older, high-quality females necessary to maintain stable populations and maintain genetic diversity.

APPENDIX 4

Table 4.1. Summary of population characteristics, life-history trait values, morphology, physiology and behavior for Painted turtles (CP; *Chrysemys picta*), Blanding's turtles (EB; *Emydoidea blandingii*), and Snapping turtles (CS; *Chelydra serpentina*) cohabiting wetlands on the Edwin S. George Reserve (ESGR). Summary data include mean (minimum-maximum), standard deviation.

Category	***Traits***	***Painted turtle (CP)***	***Blanding's turtle (EB)***	***Snapping turtle (CS)***
Population	Adult population size (stable)	550	190	200
	Adult sex ratios (M:F)	2.3:1.0	1.0:1.0	1.0:1.0
Life History	Age at maturity	9 (7-13)	17 (14-21)	13 (11-16)
	Maximum longevity	~50	>75	>55
	Male carapace length (mm)	119.5 (93-146), 9.65	200.2 (161-226),15.24	273.7 (180-367),10.13
	Female carapace length (mm)	141.1 (112-169), 9.59	198.5 (174-219),10.13	245.8 (152-300), 21.6
	Sexual size dimorphism	F > M	F = M	F < M
	Relationship - Carapace length to age	CL = 125.9 + 0.69 (age) $R^2 = 0.33$	CL = 183.5 + 0.34 (age) $R^2 = 0.10$	CL = 203.5 + 1.43 (age) $R^2 = 0.59$
	Maximum clutches per year	2/yr	1/yr	1/yr
	Reproductive frequency	1.3	0.8	0.85
	Clutch size	6.9 (2-13), 0.19	10.1 (2-19), 0.40	27.6 (12-44), 1.05
	Relationship to body size	-4.54 + 0.08 (CL) $R^2 = 0.23$	-8.58 + 0.08 (CL) $R^2 = 0.22$	-14.56 + 0.16 (CL) $R^2 = 0.27$
	Relationship to age	6.13 + 0.05(age) $R^2 = 0.06$	20.33 + 0.02(age) $R^2 = 0.04$	21.14 + 0.147 (age) $R^2 = 0.04$
	Egg width (mm)	17.33, SD = 0.22	23.72, SD = 0.16	27.62, SD = 0.29
	Relationship to body size	18.31 + 0.07 (CL) $R^2 = 0.33$	20.33 + 0.02 (CL) $R^2 = 0.04$	16.47 + 0.05 (CL) $R^2 = 0.37$
	Relationship to age	16.35 + 0.07 (age) $R^2 = 0.32$	24.07 + 0.008 (age) $R^2 = 0.07$	26.46 + 0.07(age) $R^2 = 0.15$
Behavior	Terrestrial movements	Primarily nesting	Extensive and frequent (both sexes)	Primarily nesting

Table 4.2. Predicted influence of traits on the incidence of multiple and repeat paternity assuming all other traits remain constant. Where possible, rankings are provided that demonstrate the species (CP, *Chrysemys picta*; EB, *Emydoidea blandingii*; CS, *Chelydra serpentina*), with the highest/lowest predicted multiple paternity based on the trait. Rationales are provided to describe the underlying basis for each prediction.

Table 4.2 (cont'd)

Trait	***Multiple Paternity Predictions***	***Repeat Paternity Predictions***	***Rationale for predictions***
Population			
Adult population size	CP > (CS = EB)	CP < (CS = EB)	Population size is larger in Painted turtles compared to Snapping and Blanding's turtles
Adult sex ratio (M:F)	CP > CS > EB	CP < CS < EB	Male biased sex ratio increases female encounter with males compared to even sex ratios
OSR (fluctuating)	Increase or decrease for a given year	Increase or decrease for a given year	The number of reproductive females varies in all species (Fig. 4. 1). If the number of reproductive males is constant, the operational sex ratio(OSR) will change
Life History			
Female age and size	Higher among older or larger females	Lower among older or larger females	Increased mating pressure on older or larger females may result in females mating with more males
Clutch interval	1) Higher among females with higher clutch frequencies	1) Variable with individual reproductive interval. 2) CP > (EB = CS) 3) CP only; high between clutches within a year	1) Increased mating pressure on females with high reproductive frequency may result in more opportunities to mate. 2) Longer intervals may decrease repeat paternity 3) Short receptivity window necessitates use of stored sperm
Clutch size	1) CS > EB > CP 2) Higher among females that produce more eggs per clutch	2) Lowest among females that produce more eggs per clutch	1) Clutch sizes increase ability to detect multiple paternity 2) Increased mating pressure on larger females may increase mating opportunities
Sexual size dimorphism	CS only: 1) Higher among smaller females.	1) CS only: Repeat paternity lowest among smaller females 2) CS > (EB = CP)	1) Males larger than females; multiple paternity may increase among smallest females 2) Proposed male-dominance system can result in females re-encountering the same male
Behavior			
Terrestrial movement	1) EB > (CS = CP) 2) EB < (CS = CP)	1) EB < (CS = CP) 2) EB > (CS = CP)	1) Extended terrestrial movements increases encounters with non-resident individuals 2) Re-visiting the same habitats can increase encounters between the same individuals

Table 4.3. Primary relationship between measures of female qualities and age or size that have implications for male reproductive success in Painted turtles (CP; *Chrysemys picta*), Blanding's turtles (EB; *Emydoidea blandingii*), and Snapping turtles (CS; *Chelydra serpentina*).

Relationships	**CP**	**EB**	**CS**
Inter-annual reproductive frequency and age	Positive	Positive	Positive
Intra-annual reproductive frequency and age	Positive	NA	NA
Egg width and age	Positive	Positive	Positive
Egg width and carapace length	Positive	No	No
Clutch size and carapace length	Positive	Positive	Positive

Table 4.4. Comparison of among year reproductive intervals of females based on long-term data (>33 years) for Painted turtles (CP), Blanding's turtles (EB) and Snapping turtles (CS).

Species	Reproductive Frequency	Mean	Min-Max	SD
CP	Among-year interval	1.47	1-4	0.44
EB	Among-year interval	1.66	1-4	0.55
CS	Among-year interval	1.66	1-3	0.49

Table 4.5. Comparisons of the direction and magnitude of effects of factors influencing multiple paternity in Painted turtles, Blanding's turtles, and Snapping turtles (2003-2006).

	Painted turtles			***Blanding's turtles***			***Snapping turtles***		
	Beta estimate	**Z-value**	**p-value**	**Beta estimate**	**Z-value**	**p-value**	**Beta estimate**	**Z-value**	**p-value**
Female age	0.115	2.01	0.044	0.032	0.98	0.328	0.087	2.67	0.008
Clutch size	-0.046	-0.32	0.752	0.476	2.51	0.012	0.033	0.78	0.433
Female body size	-0.011	-0.23	0.819	0.074	1.90	0.058	0.051	2.62	0.009

Table 4.6. Comparisons of factors influencing male reproductive success (total number of offspring) for successful Painted turtle and Blanding's turtle males on the ESGR (2003-2006).

	Painted turtles			***Blanding's turtles***		
	Beta estimate	**t-value**	**p-value**	**Beta estimate**	**t-value**	**p-value**
Total number of mates	3.47	1.18	0.242	7.95	4.36	<0.001
Total number of clutches	5.82	10.24	<0.001	5.40	8.07	<0.001
Average offspring sired per clutch	1.60	4.25	<0.001	0.91	1.73	0.096
Average multiple paternity	-4.05	-1.90	0.062	-4.58	-2.35	0.027
Male age (average)	-0.29	-1.13	0.272	0.05	0.08	0.934
Male size (average)	0.04	0.87	0.387	0.10	0.08	0.627

Fig. 4.1. Summaries of the a) number, b) body sizes and c) ages of reproductive female Painted turtles (*Chrysemys picta*), Blanding's (*Emydoidea blandingii*) and Snapping turtles (*Chelydra serpentina*) cohabiting wetlands on the Edwin S. George Reserve each year from 1977-2006.

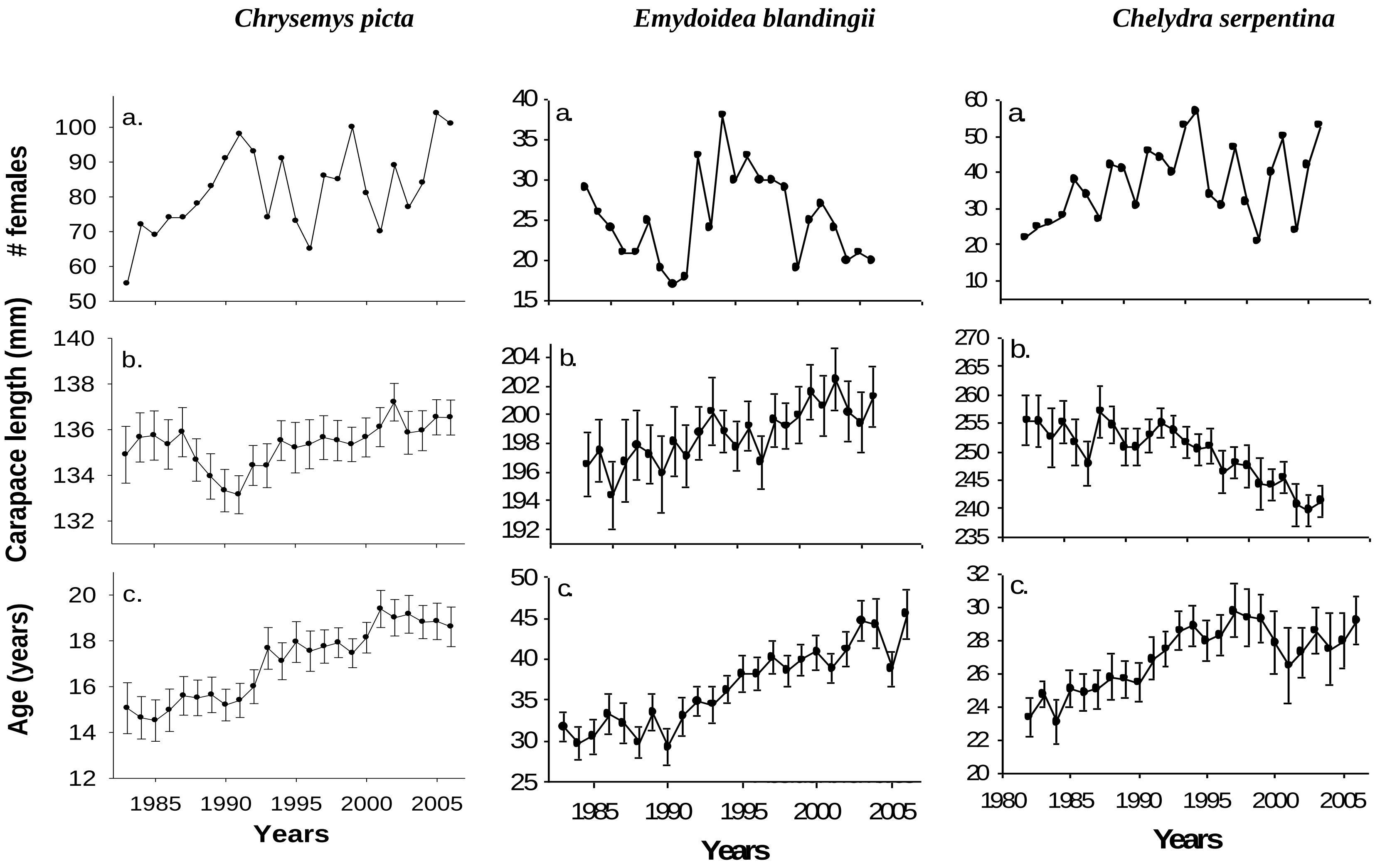


Fig. 4.2: Conceptual diagram illustrating how population, life-history, morphology and behavior can directly (solid arrows) and indirectly (dashed arrows) influence male reproductive success.

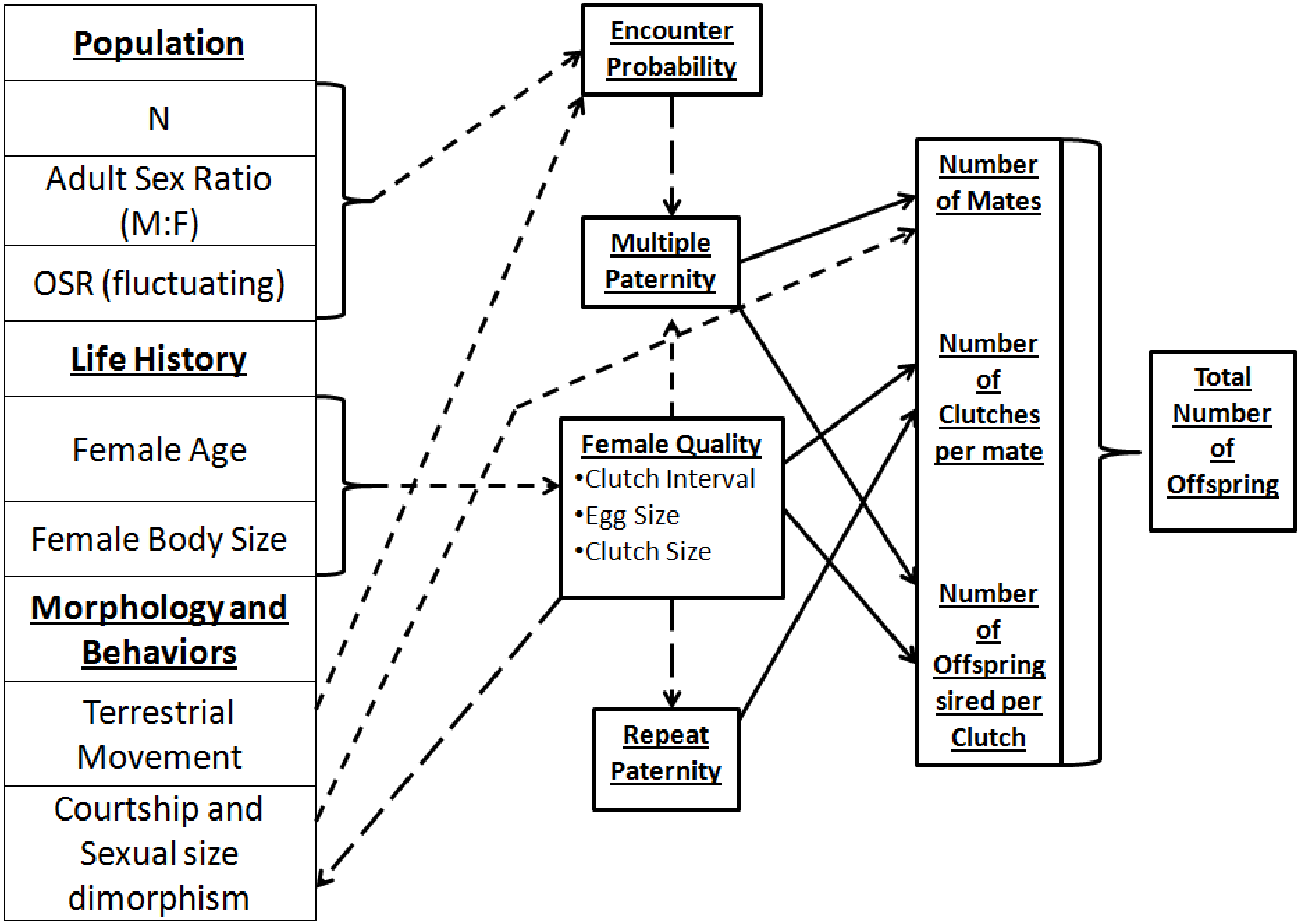
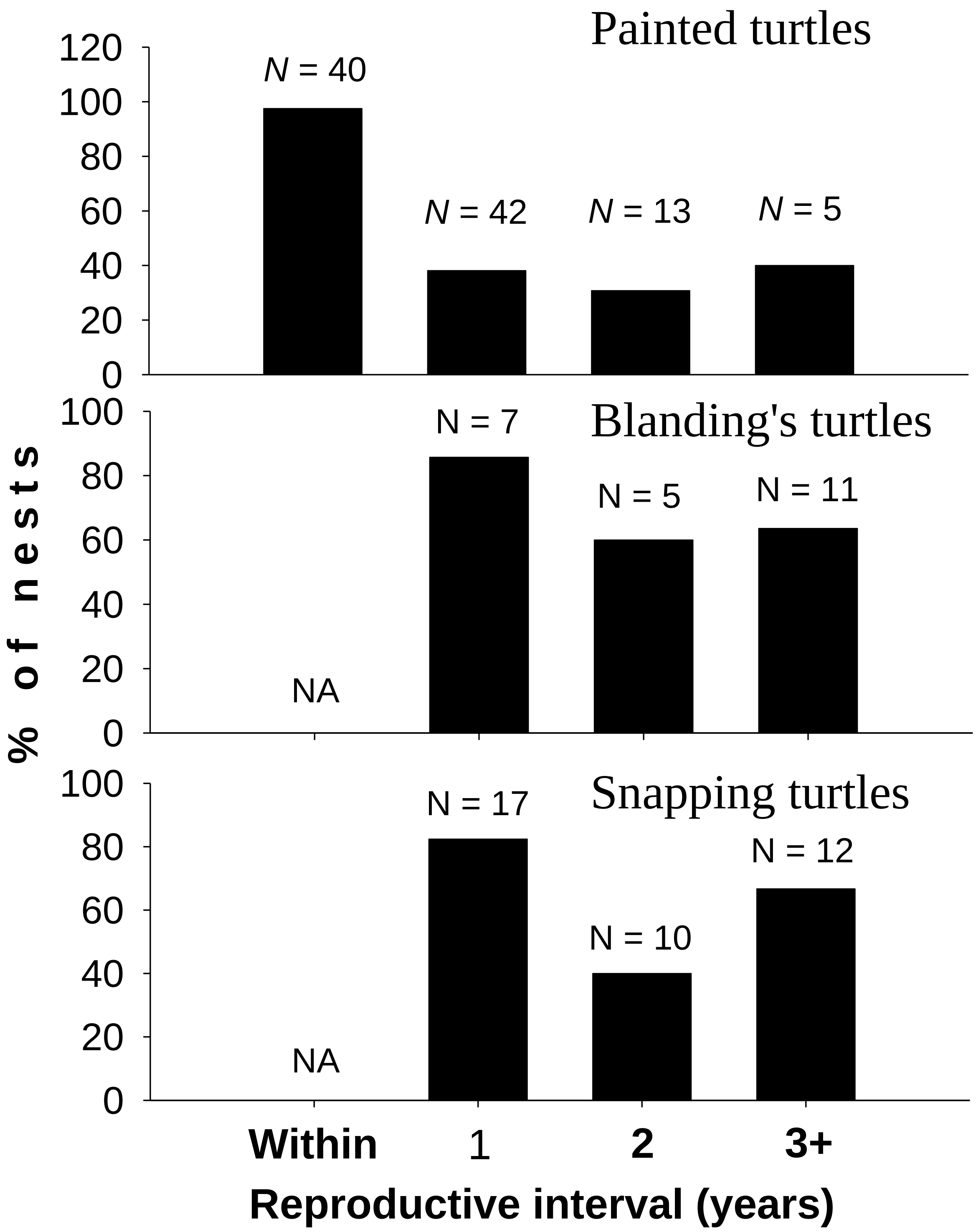


Fig. 4.3. Percent of clutches showing evidence of repeat paternity (and sample sizes; N) within and among years for Painted turtles, Blanding's turtles, and Snapping turtles.



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